Investigating Copper toxicity in Caenorhabditis elegans

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Abstract

Increases in global industrialisation are causing a growing release of pollution into the environment. Pollution, particularly by heavy metals is a significant problem as they are non-biodegradable, thus able to accumulate in ecological systems. The heavy metal copper (Cu) though toxic in excess is also an essential trace element that serves as a cofactor in many critical biological processes such as respiration, iron transport and oxidative stress protection.

It is therefore important to assess the effects of Cu toxicity on the ecosystem and its natural communities, as environmental pollution impacts on an organism's genomic utilisation resulting in consequences for its biology and thus ultimately affecting population dynamics. Therefore the overall objective of this project was to investigate Cu homeostasis and toxicity using the soil dwelling nematode, *Caenorhabitis elegans* as a model organism.

The effects of Cu toxicity on the population dynamics of *C. elegans* was determined by investigating changes in life cycle traits. The whole organism response to Cu toxicity was investigated in *C. elegans* and the EC50 and LC50 of $CuSO_4$ was determined, along with the effects of Cu on growth and development. Cu tolerant mutants were created and phenotypic effects examined among them in order to assess adaptive responses to heavy metal exposure.

To enhance our understanding of the complexities of Cu homeostasis at the genetic level the expression profile and functional significance of two putative Cu transporters Ctr and CutC were analysed. Using QPCR technology both genes were found to be down regulated with increasing $CuSO_4$ concentrations. RNA-mediated interference (RNAi) technology was exploited to create knockdowns of Ctr and CutC which resulted in significant differences in the *C. elegans* life cycle in the presence and absence of Cu.

Overall RNAi of Ctr and CutC resulted in different demographic and phenotypic effects in *C. elegans*, indicating at their different roles in Cu metabolism, with Ctr postulated to be a high affinity Cu importer and CutC possibly with a complex role in regulation of Cu proteins. In Summary Cu homeostatis is a complex process maintained by the interactions of many interconnected, but also independent components to ensure that concentrations of this essential yet toxic element are tightly controlled.

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This thesis is dedicated to my parents, Efra and Godwin.
Without their emotional and financial support
none of this would have been possible.

'...to myself I seem to have been only like a boy playing on the sea-shore, diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me...'

Brewster's Memoirs of Newton. Vol. II. Chap. XXVII
***Sir Isaac Newton** (1642-1727)*

Contents

Chapter 2: Materials and Methods

Abbreviations

AA	- Amino acids
AAS	- Atomic Absorption Spectrophotometry
A_x	- Absorbance at x nm
Amp	- Ampicillin
AP	- Alkaline phosphatase
ATP	- Adenosine 5`-triphosphate
BLAST	- Basic Local Alignment Search Tool
bp	- base pair
BSA	- Bovine serum albumin
Carb	- Carbenicillin
cDNA	- Complementary deoxyribonucleic acid
C. elegans	- *Caenorhabitis elegans*
CIAP	- Calf intestinal alkaline phosphatase
Cm	- Chloramphenicol
CMGP	- Cartilage matrix glycoprotein
Cu	- Copper
D. melanogaster	- *Drosophila melanogaster*
dH_2O	- distilled water
DNA	- Deoxyribonucleic acid
dNTPs	- Adenosine, cytidine, guanosine and thymidine 5`deoxyribonucleotide triphosphates
dsDNA	- Double stranded deoxyribonucleic acid
dT	- Oligonucleotide of thymidine residues
DTT	- Dithiothreitol
E. coli	- *Escherichia coli*
EDTA	- Ethylenediaminetetracetic acid
EST	- Expressed sequence tag
EtBr	- Ethidium bromide
Fe	- Iron
GFP	- Green fluorescent protein

GSH	- Glutathione
H. sapiens	- *Homo sapiens*
HPLC	- High Performance Liquid Chromatography
IPTG	- Isopropyl β-D-thiogalactopyranoside
Kb	- Kilo base pairs (1000 base pairs)
λH3	- Lambda *Hind*III Digest
LB	- Luria Bertani
LB+X	- Luria broth containing antibiotic denoted by X.
Kan	- Kanamycin
M. musculus	- *Mus musculus*
M-MLV RT	- Moloney murine leukemia virus reverse transcriptase
mRNA	- Messenger ribonucleic acid
MT	- Metallothionein
O/N	- Overnight
PCR	- Polymerase chain reaction
Poly (A^+)	- Polyadenylated 3′ terminus
ppm	- Parts per million
psi	- Pounds per square inch
R. norvegicus	- *Rattus norvegicus*
RNA	- Ribonucleic acid
RNasin	- Ribonuclease inhibitor
RNase	- Ribonuclease
RNAi	- RNA interference
rRNA	- Ribosomal ribonucleic acid
RT	- Reverse transcriptase
R/T	- Room temperature
S. cerevisiae	- *Saccharomyces cerevisiae*
SDS	- Sodium dodecyl sulphate
ssDNA	- single stranded deoxyribonucleic acid
SOD	- Superoxide dismuatse
TAE	- Tris:acetic acid:EDTA
Taq	- *Thermus aquaticus*
TE	- Tris-EDTA buffer

T_m	- Melting temperature
Tris	- 2-Amino-2-(hydroxymethyl)-1,3-propanediol
U	- Enzyme units
X^R	- Resistance to antibiotic (X)

Chapter 1

General Introduction

Chapter 1

General Introduction

1.1 Pollution

There is an increasing level of concern about environmental contaminants and their effects on human health and the surrounding ecosystems. The deterioration of environmental quality has existed as a serious problem under the ever-increasing impacts of an exponentially growing population and an industrialising society (Nriagu, 1979). Increases in global industrialisation have caused a growing release of pollution into the environment. Industrialisation transforms the chemical composition of our environment, creating novel compounds and redistributes natural resources. This pollution now affects the whole biosphere, influencing its components that include terrestrial, fresh-water and marine ecosystems (Nriagu and Pacyna, 1998).

On a global scale, hazardous waste production was 400 million tonnes a year in the early 1990s, with 50-70% of it disposed on land (UNEP, 1994). Environmental contamination of air, water, soil and food has become a threat to the continued existence of many plant and animal communities in our ecosystem and may ultimately threaten the very survival of the human race (Nriagu, 1979).

1.2 Heavy metals

There are various types of pollution, both natural and synthetic, which affect the environment in different manners. One of the major groups of xenobiotics released into the environment are the inorganic heavy metals. The term "heavy metals" describes elements in the periodic table that have a relative density of at least five times that of water (Nieboer and Richardson, 1980). Thus, heavy metals are commonly defined as those having a specific density of more than $5g/cm^3$ (Järup, 2003).

Metals are natural substances found in the environment and some are essential co-factors for life. Essential heavy metals vary from species to species but a core of five, Iron (Fe), Copper (Cu), Zinc (Zn), Manganese (Mn) and Molybdenum (Mo), are required by most organisms. Others such as Nickel (Ni), Cobalt (Co) and

Vanadium (V) are required by specialised enzymes in a smaller sub-set of species and finally Tungsten (W) is known or suspected to have essential roles in at least one species (Cavet, *et al.*, 2003).

1.3 Heavy metal pollution

Heavy metals can be released into the environment by both natural sources and human activities. The natural biogeochemical cycle of heavy metals mainly consists of wind-borne soil particles, sea-salt spray, volcanoes, forest fires and decaying vegetation (Moolenaar and Lexmond, 1999; Lenntech, 2004). However, as heavy metals have been used by humans for thousands of years, the root of most sources is through human activity (Järup, 2003). The main anthropogenic sources of heavy-metal emissions are mining and smelting of metal ores, incineration plants, automobile exhausts and fossil fuel combustion (Moolenaar and Lexmond, 1999).

Although metals are naturally occurring chemicals, human influences have altered their distribution in the environment (van Geen and Luoma, 1999). By extracting metals from ores, manipulating their chemical speciation in industry, and dumping them into the environment in altered forms, humans have disrupted the natural processes that govern the fate of metals (Waalkes, 1995).

The adverse health effects of heavy metals has been known for a long time, yet exposure to heavy metals continues, and is even increasing in some less developed parts of the world. Since the middle of the 19th century, production of heavy metals has increased steeply with concomitant emissions to the environment (Järup, 2003). Nriagu and Pacyna (1998) stated that the man-induced mobilisation of heavy metals into the biosphere is greatly increasing the circulation of toxic metals through soil, water and air.

Emissions of heavy metals to the environment occur via a wide range of processes and pathways, including to the air (e.g. during combustion, extraction and processing), to surface waters (via runoff and release from storage and transport) and to the soil (and hence into ground waters and crops) (Järup, 2003). The movement of heavy metals through the environment is shown in ***Figure 1.1***.

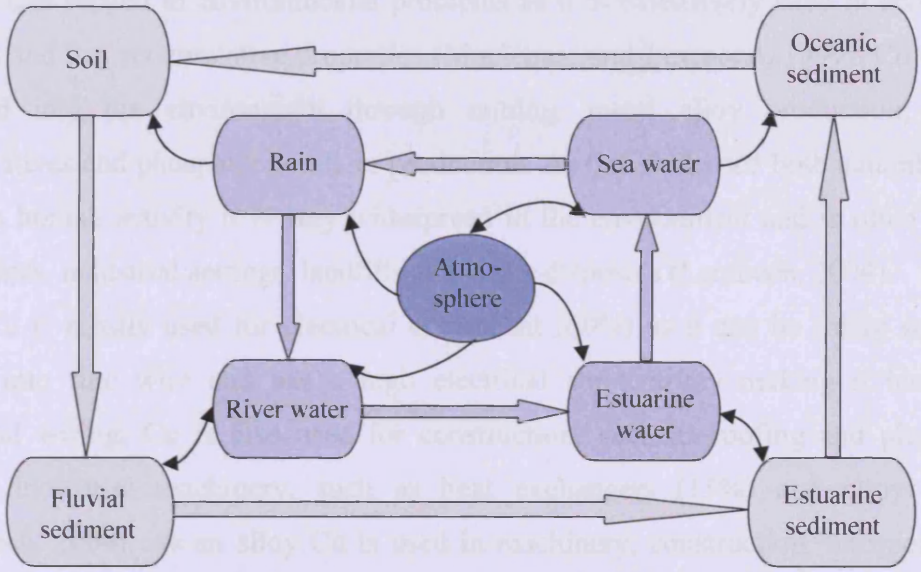

Figure 1.1 *Movements of heavy metals through the hydrocycle. The outer ring represents the particulate transport, the inner ring the movement of soluble trace elements and the centre indicates the influence of the atmosphere on the entire cycle (Adapted from Salomons and Forstner, 1984).*

In the past decades, both terrestrial and aquatic environments have been polluted with metals (Hopkin, 1989). Polluted river water causes polluted sediments which when deposited on land pose a potential threat to terrestrial ecosystems. Metals in the soil can be taken up by plants and transferred to a higher trophic level by means of herbivory (Notten *et al.*, 2005). As heavy metals are non-biodegradable they have a tendency to accumulate in the environment. This leads to heavy metals occurring at concentrations that are abnormal and therefore adversely affecting the habitat they contaminate (Leeper, 1978).

One important heavy metal that is ubiquitous in our modern technological environment is copper (Cu). Though Cu is essential for the function of living organisms it can accumulate in ecological systems and while low exposure to Cu is not harmful, exposure to elevated concentrations of Cu is damaging.

1.4 Copper in terrestrial ecosystems

Cu is related to environmental problems as it is extensively used in economic process and has accumulative properties (Moolenaar and Lexmond, 1999). Cu can be released into the environment through mining, metal alloy production, wood preservatives and phosphate fertilizer production. As Cu is released both naturally and through human activity it is very widespread in the environment and is often found near mines, industrial settings, landfills and waste disposals (Lenntech, 2004).

Cu is mostly used for electrical equipment (60%) as it can be easily worked, drawn into fine wire and has a high electrical conductivity making it ideal for electrical wiring. Cu is also used for construction, such as roofing and plumbing (20%); industrial machinery, such as heat exchangers (15%) and alloys (5%) (Lenntech, 2004). As an alloy Cu is used in machinery, construction, transportation and military weapons (Barceloux, 1999; Winge and Mehra, 1990) and is an important component of white gold and other alloys used for imitation jewellery (Vilaplana *et al.*, 1991). Cu is also used in dental products (Lucas and Lemons, 1992) and in cosmetics (Gaetke and Chow, 2003). The world's Cu production is still rising with increasing amounts being released into the environment (Barceloux, 1999).

Cu enters the soil from atmospheric deposition and from different soil amendments and as a result soils may contain large quantities of Cu (Moolenaar and Lexmond, 1999). Cu enters the air mainly through release during the combustion of fossil fuels. It will remain there for an eminent period of time, before it settles when it starts to rain and then ends up mostly in soils (Lenntech, 2004). Cu can also be introduced into soils via sewage sludge, mine effluents, industrial waste, commercial fertilisers, liming materials, pesticides, manures and compost (Barceloux, 1999; Moolenaar, and Lexmond, 1999).

For more than 100 years Cu has been used in agriculture as the active ingredient in bacteriocides and fungicides on fruits and vegetables (Brown *et al.*, 1992). As Cu does not break down in the environment it can accumulate in plants and animals. On Cu-rich soils only a few plant species are capable of survival, therefore plant diversity near Cu-disposing factories is limited. Due to the effects upon plants Cu is a serious threat to farmlands. Cu can seriously influence the production of certain farmlands, depending upon the acidity of the soil and the presence of organic matter. Despite of this, Cu-containing manures are still applied (Lenntech, 2004).

In terrestrial ecosystems Cu is adsorbed on negative surfaces in soils like other heavy metals. The oxidation state of a heavy metal relates to the solubility and toxicity of the metal itself (Spain and Alm, 2003). Cu strongly associates with organic matter and moves as an anion in organic combination or as a neutral molecule in soil. Chelated Cu though less toxic than the cation, is more bio-available to soil-dwelling species (Leeper, 1978). Cu interrupts the natural proceedings in soils, as it negatively influences the activity of microrganisms and earthworms. The decomposition of organic matter can be seriously slowed down because of this. Moreover, Cu can inhibit soil organisms (Alexander *et al.*, 1999) and may accumulate in certain species such as earthworms having detrimental effects along the rest of the food chain (***Figure 1.2***).

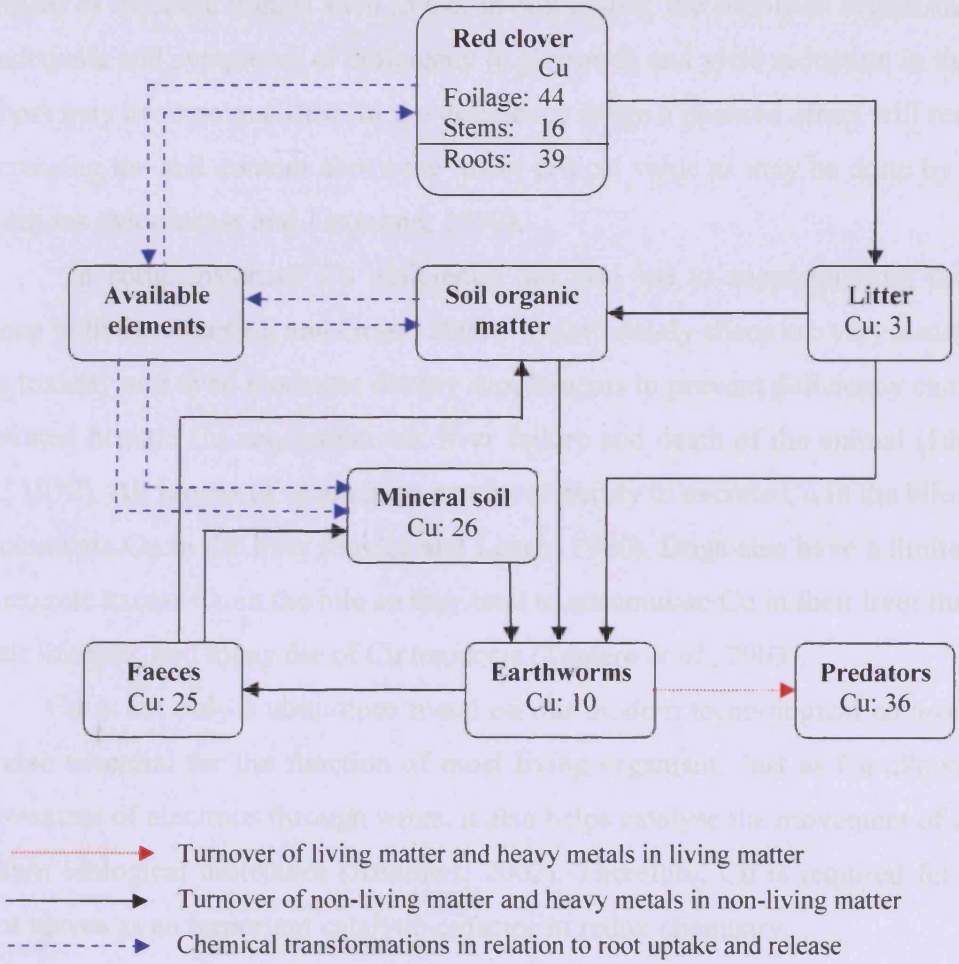

Figure 1.2. *Heavy metal concentrations (mg kg^{-1} dry weight) in earthworms, their surroundings and predators (Satchell, 1983).*

Transfer of metals to various food chain compartments is influenced by many factors, and soil pH, organic matter and clay content, highly influence metal speciation and thus bioavailability (Notten *et al.*, 2005). Unlike the majority of organic chemicals that can be eliminated from tissues by metabolic degradation, the metals as elements are indestructible and therefore have the potential for accumulation along the food chain (Moolenaar and Lexmond, 1999). Food plants that tolerate relatively high concentrations of potentially hazardous metals create a greater health risk to their consumers than those that are more sensitive. Excessive uptake of Cu may result in adverse effects on soil biota, plants and due to transfer via the food chain, on mammals, birds and human consumers (Moolenaar and Lexmond, 1999).

A paradox exists however, as many soils may also be deficient in essential metals such as Cu, resulting in mineral disorders that affect livestock at pasture. If the content of essential metals such as Cu, in soil is low, the supply to organisms will be inadequate and symptoms of deficiency (e.g. growth and yield reduction in the case of crops) may become manifest. In the deficiency range a positive effect will result from increasing the soil content above the lower critical value as may be done by fertiliser additions (Moolenaar and Lexmond, 1999).

In some instances Cu deficiency has also led to supplementing the diet of sheep with Cu (Rensing and Grass, 2003). Unfortunately sheep are very susceptible to Cu toxicity and even moderate dietary supplements to prevent deficiency can result in elevated hepatic Cu concentrations, liver failure and death of the animal (Ishmael, *et al.*, 1972). All breeds of sheep have a reduced ability to excrete Cu in the bile and thus accumulate Cu in the liver (Saylor and Leach, 1980). Dogs also have a limited ability to excrete excess Cu in the bile so they tend to accumulate Cu in their liver throughout their lifespan, and many die of Cu toxicosis (Tapiero *et al.*, 2003).

Cu is not only a ubiquitous metal on our modern technological environment, it is also essential for the function of most living organism. Just as Cu allows for the movement of electrons through wires, it also helps catalyse the movement of electrons within biological molecules (Andrews, 2002). Therefore, Cu is required for survival as it serves as an important catalytic cofactor in redox chemistry.

1.5 Copper homeostasis

Cu ions can adopt distinct redox states, oxidised Cu^{2+} or reduced Cu^{+}, allowing the metal to play a pivotal role in cell physiology as a catalytic cofactor in the redox chemistry of enzymes and proteins (Tapiero *et al.*, 2003). These enzymes and proteins carry out fundamental biological functions that are required for growth and development (Linder, 1991). Cu proteins are involved in vital processes such as respiration (mitochondrial oxidative phosphorylation), iron transport, oxidative stress protection (free radical scavenging), blood clotting, hormone production, neurotransmitter synthesis and maturation, elastin cross-linking and pigmentation (Petris *et al.*, 2003; Puig *et al.*, 2002; Tapiero *et al.*, 2003). As Cu requiring proteins are involved in such a wide variety of biological processes, deficiency in these enzymes or alteration in their activities, often cause disease states or pathophysiological conditions (Pena *et al.*, 1998).

Though if present in excess, free Cu ions can cause damage to cellular components because of its redox activity and a delicate balance between the uptake and efflux of Cu ions determines the amount of cellular Cu (Tapiero *et al.*, 2003). Intracellular Cu concentrations need to be regulated within very narrow limits (Rensing and Grass, 2003) and several families of proteins are emerging that control the activity of intracellular metal ions and help confine them to vital roles. These include integral transmembrane transporters, metalloregulatory sensors and diffusible cytoplasmic metallochaperone proteins that protect and guide metal ions to targets (Finney and O'Halloran, 2003).

1.6 Cuproenzymes and metalloproteins

Ingested Cu is absorbed and distributed to various Cu-requiring proteins. Cu is an essential cofactor for a number of enzymes called cuproenzymes which are involved in a diverse range of biological processes for growth, development and maintenance (Gaetke and Chow, 2003). A list of Cu requiring enzymes and their functions are shown in ***Table 1.1***.

ENZYME	FUNCTION
Cytochrome-c oxidase	Electron transport in mitochondria
Cu/Zn-SOD	Free radical detoxification
Metallothionein	Storage of excess Cu and other divalent metal ions [not Fe^{2+}]. Possible donor of Cu to certain apoproteins
Ceruloplasmin (extracellular)	Ferroxidase, promotes flow of Fe from liver to blood scavenger of reactive oxygen species (ROS), acute-phase reactant. Cu transport
Lysyl oxidase	Formation of embryonic structures
Protein-lysine-6-oxidase	Cross-linking of collagen and elastin
Tyrosine (catechol oxidase)	Formation of melanin
Dopamine-β-monooxygenase	Catecholamines production
α-Amidating enzyme	Modifies C-terminal ends of hypothalamic peptide hormones ending in glycine, leaving the COOH of the next to last amnio acid amitdated (necessary for hormone maturation)
Diamine oxidase	Inactivation of histamine and polyamines (cellular and extracellular)
Amine oxidase (extracellular)	Inactivation of histamine, tyramine, dopamine, serotonin?
Peptidylglycine monooygenase	Bioactivation of peptide hormones
Hephaestin	Ferroxidase, in trans-golgi of enterocytes; aids iron absorption homology to ceruloplasmin
CMGP	Ferroxidase/amine oxidase, homologous to ceruloplasmin (chondrocytes and eye ciliary epithelia)
β-Amyloid precursor protein	Normal function currently unknown
Prion protein (PrPC)	Cu binding properties suggest that it may protect against ROS; has SOD-like activity; may return Cu to neurones at synapses (many cells)
S-Adenosylhomocysteine	Sulphur amino acid metabolism hydrolase
Angiogenin	Induction of blood vessel formation
Blood clotting factors V/ VIII	Blood clotting

Table 1.1 *Copper-dependant enzymes in mammals* (adapted from Tapiero *et al.*, 2003; Andrews, 2002).

Non-enzymic metalloproteins function in storage, transport and/or detoxification systems. These Cu metalloproteins represent a new family of soluble, low molecular weight proteins that function to deliver Cu to specific sites within a cell (Portnoy *et al.*, 2001). Metalloproteins are normally found only in traces but exposure to sub-lethal amounts of heavy metals induces increased synthesis.

1.7 Extracellular copper transport

All living organisms, from bacteria to humans, require dietary Cu for continued growth and development. This nutritional requirement stems from the essential role of Cu in the function of the numerous cuproproteins (Vulpe and Packman, 1995). Given that as many as two billion people suffer from malnutrition due to deficiencies of micronutrients such as Cu (Eide, 1998), it highlights the necessity of organisms to obtain essential metal ions from its diet to maintain its health. Based on genetic and biochemical investigations, the molecular mechanisms of trace metal uptake have been shown to be, at least for Cu, largely conserved mechanisms across the animal kingdom (Rees and Thiele, 2004).

1.7.1 Extracellular *copper uptake*

The main sources of dietary Cu are seeds, grains, nuts and beans (concentrated in the germ and bran), dried fruit, shellfish and liver (Tapiero *et al.*, 2003). Thus, Cu intake varies greatly depending on food choices and dietary customs. The average intake of Cu by human adults ranges from 0.6 to 1.6 mg/d, and amounts that are considerably higher are usually not problematic for humans or rodents. Even daily intakes of Cu as great as 3mg/d for children and 8mg/d for adults are considered tolerable (Tapiero *et al.*, 2003). Though individuals with inherited propensities to accumulate Cu are vulnerable to toxicity from ingestion at these higher doses (Montaser *et al.*, 1992).

Biological management of Cu requires active uptake from the environment through the epithelia barrier of the intestine, safe transport in extracellular fluids and delivery to cells that utilise the metal (Andrews, 2001). The absorption of Cu in the human body depends on a variety of factors including chemical form and presence of other dietary components. About 30-50% of ingested Cu, mostly as Cu^{2+}, is absorbed in the small intestine with very small amounts being absorbed in the stomach (Turnlund *et al.*, 1997). In humans with normal intakes, Cu is absorbed and actively recycled between the digestive tract, body fluids and tissues (particularly the liver). Thus dietary Cu contributes only a small proportion of the total reabsorbed from saliva, gastric juices, the bile, pancreatic and duodenal fluids (Tapiero *et al.*, 2003).

Amino acids, particularly histidine, methionine and cysteine, bind to Cu to allow absorption through an amino acid transport system. Reduced glutathione (GSH)

and organic acids, such as citric, gluconic, lactic and acetic acids, form ligands with Cu that are also readily absorbed (Jacob *et al.*, 1987). Within mucosal cells, most newly absorbed Cu (about 80%) is retained in the cytosol, bound to GSH, metallothioneins and/or proteins of similar size to prevent cytotoxicity (Tapiero *et al.*, 2003).

1.7.2 Cellular copper uptake

Two well defined Cu uptake pathways are used by mammalian cells, Cu bound to ceruloplasmin and Cu not bound to ceruloplasmin. These two systems allow entry of Cu into the cell via initially distinct but ultimately convergent paths as shown in ***Figure 1.3*** (Vulpe and Packman, 1995).

Cu absorbed from the small intestine is transported in the blood bound predominantly to ceruloplasmin for transport to tissues, but may also bind to albumin, transcuperin and histidine. Ceruloplasmin is the most abundant Cu protein and contains 70-95% of plasma Cu. Each ceruloplasmin protein tightly binds 6 or 7 Cu atoms with a variety of Cu-binding sites and transports the Cu to cells and intracellular Cu proteins. At least half of the Cu from the ceruloplasmin first enters a membrane-enclosed space such as endosomes (***Figure 1.3***). Radioactive Cu can be tracked from this compartment and later appears in Cu proteins such as SOD (Vulpe and Packman, 1995).

Free Cu uptake occurs via two different systems; the intestinal mucosa apical transport and via the Hepatocytes and fibroblasts. Entry of free Cu into the cell is facilitated by passive transporters and follows a path different from that of Cu bound to ceruloplasmin (***Figure 1.3***). Cu routed through the two uptake systems, Ceruloplasmin-mediated and free-Cu uptake, at some point converge into a common path. Most non-hepatic cells are likely to possess both pathways and in studies it was found that free Cu inhibited uptake of Cu from ceruloplasmin and vice versa (Vulpe and Packman, 1995).

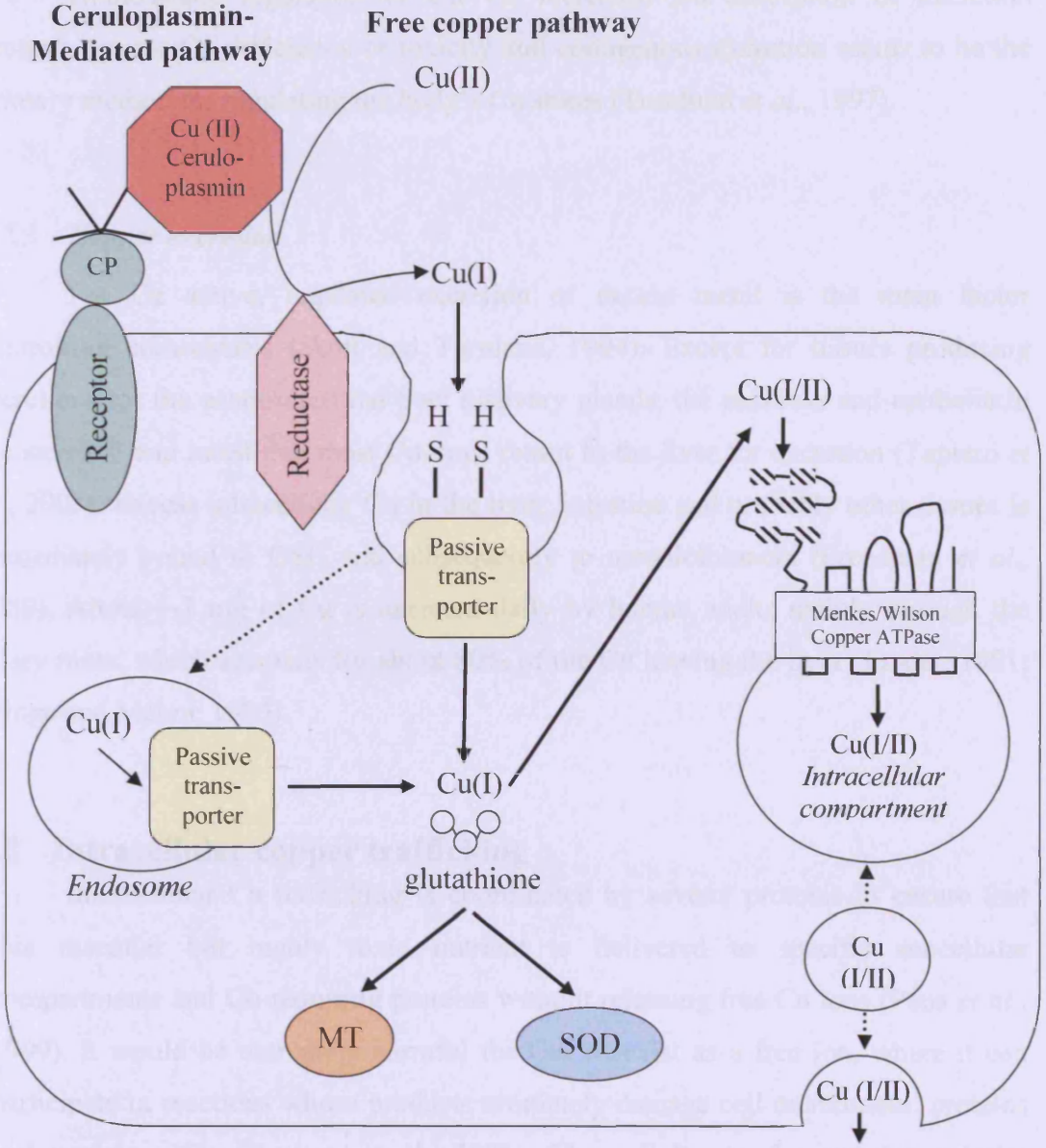

Figure 1.3 *Diagram showing a unified model of Cu uptake through the ceruloplasmin and free Cu pathway. In ceruloplasmin-mediated transport, binding to a ceruloplasmin receptor at the cell surface is followed by release of Cu from cerluoplasmin in a step requiring Cu reduction. Through the free Cu pathway, Cu(I) enters the cytoplasm via a passive transport protein that does not require cellular energy to function. Some Cu transported into the cytosol occurs at the cell surface, and some occurs after internalisation of the Cu-transporter complex via an endosome. Cu(I)-Glutathione transfers cytosolic Cu to cuproproteins, such as MT or SOD. Cu is delivered to the export system by the Menkes or Wilson Cu-transporting ATPase in one or more intracellular compartments or organelles. In this model, free Cu or Cu bound to other ligands utilises the same reductase and follows the same path as Cu from ceruloplasmin (adapted from Vulpe and Packman, 1995).*

Homeostatic regulation of Cu via increased Cu absorption or excretion protects against Cu deficiency or toxicity and endogenous excretion seems to be the primary method for regulating the body's Cu stores (Turnlund *et al.*, 1997).

1.7.3 Copper excretion

For Cu active, regulated excretion of excess metal is the main factor controlling homeostasis (Scott and Turnland, 1994). Except for tissues producing secretions for the gastrointestinal tract (salivary glands, the pancreas and epithelia in the stomach and intestine), most Cu must return to the liver for excretion (Tapiero *et al.*, 2003). Excess intracellular Cu in the liver, intestine and probably other tissues is immediately bound to GSH and subsequently to metallothionein (Freedman *et al.*, 1989). About 1-2 mg of Cu is excreted daily by human adults mainly through the bilary route, which accounts for about 80% of the Cu leaving the liver (Linder, 1991; Winge and Mehra, 1990).

1.8 Intracellular copper trafficking

Intracellular Cu trafficking is coordinated by several proteins to ensure that this essential but highly toxic nutrient is delivered to specific subcellular compartments and Cu-requiring proteins without releasing free Cu ions (Pena *et al.*, 1999). It would be extremely harmful for Cu^{+} to exist as a free ion, where it can participate in reactions whose products ultimately damage cell membranes, proteins and nucleic acids (Tapiero *et al.*, 2003). The cellular uptake and intracellular distribution of Cu is therefore a precisely orchestrated process. The components that are involved in intracellular Cu trafficking and their roles in Cu homeostasis have been identified by studies on model organisms such as *Saccharomyces cerevisiae* and *Escherichia coli* and by characterisation of the molecular basis for Cu-related genetic disorders in humans (Lee *et al.*, 2002).

Cu metabolism is maintained by proteins that transport metals into cells (importers), that transport metals out of cells (exporters), that change the oxidation state of metal ions to assist in their transport (reductases and oxidases) and that are involved in intracellular Cu distribution and utilisation (metallochaperones). The identification of mammalian homologues of these proteins reveals a remarkable

structural and functional conservation of Cu metabolism between bacteria, yeast and humans (Pena *et al.*, 1999). ***Figure 1.4*** shows a diagram of Cu trafficking in yeast, humans and nematodes and ***Table 1.2*** lists the proteins involved.

1.8.1 Intracellular Copper chaperones

As Cu ions are compartmentalised into different subcellular organelles intracellular transport systems are required (Eide, 1998). Cu and Cu proteins are distributed throughout the cell and in all cellular organelles, including the nucleus, mitochondria, lysosomes, endoplasmic reticulum and cytosol. One fourth to one half of cellular Cu is cytosolic. Slightly less Cu is present in the nucleus, and a smaller but nonetheless significant amount is found in the mitochondria and endoplasmic reticulum (Vulpe and Packman, 1995).

The processes controlling the distribution of Cu to different cellular compartments and its delivery to Cu proteins are poorly understood. The known Cu chaperones are divided into three functional groups: the Atx1-like chaperones, the Cu chaperones for superoxide dismutase (SOD) and the Cu chaperones for cytochrome C oxidase (Prohaska and Gybina, 2004). Cu chaperones that have been identified in yeast, humans and nematodes are shown in ***Figure 1.4*** and ***Table 1.2***.

Metal-trafficking proteins must bind their cargo ions tightly enough to prevent adventitious reactions or release of the ions, but this coordination environment must also allow for easy transfer of the metal to the target (Finney and O'Halloran, 2003). Following entry, the detoxification mechanisms found across species include the binding of Cu to specific proteins (e.g. metallothioneins), the transfer of Cu into isolated cell compartments (e.g. periplasmic space, lysosome) and the export of Cu from the cell (Vulpe and Packman, 1995).

In principle, disruption of any of these steps could lead to defects in metal balance and consequent clinical disorders (Andrews, 2002). There must exist a responsive form of communication between the proteins responsible for Cu homeostasis, the organelles in which they reside, the external environment and perhaps other cellular processes (Rees and Thiele, 2004). Many of the genes and proteins that orchestrate Cu metabolism are regulated by intracellular Cu concentrations, either at the level of transcription, protein localisation, or stability.

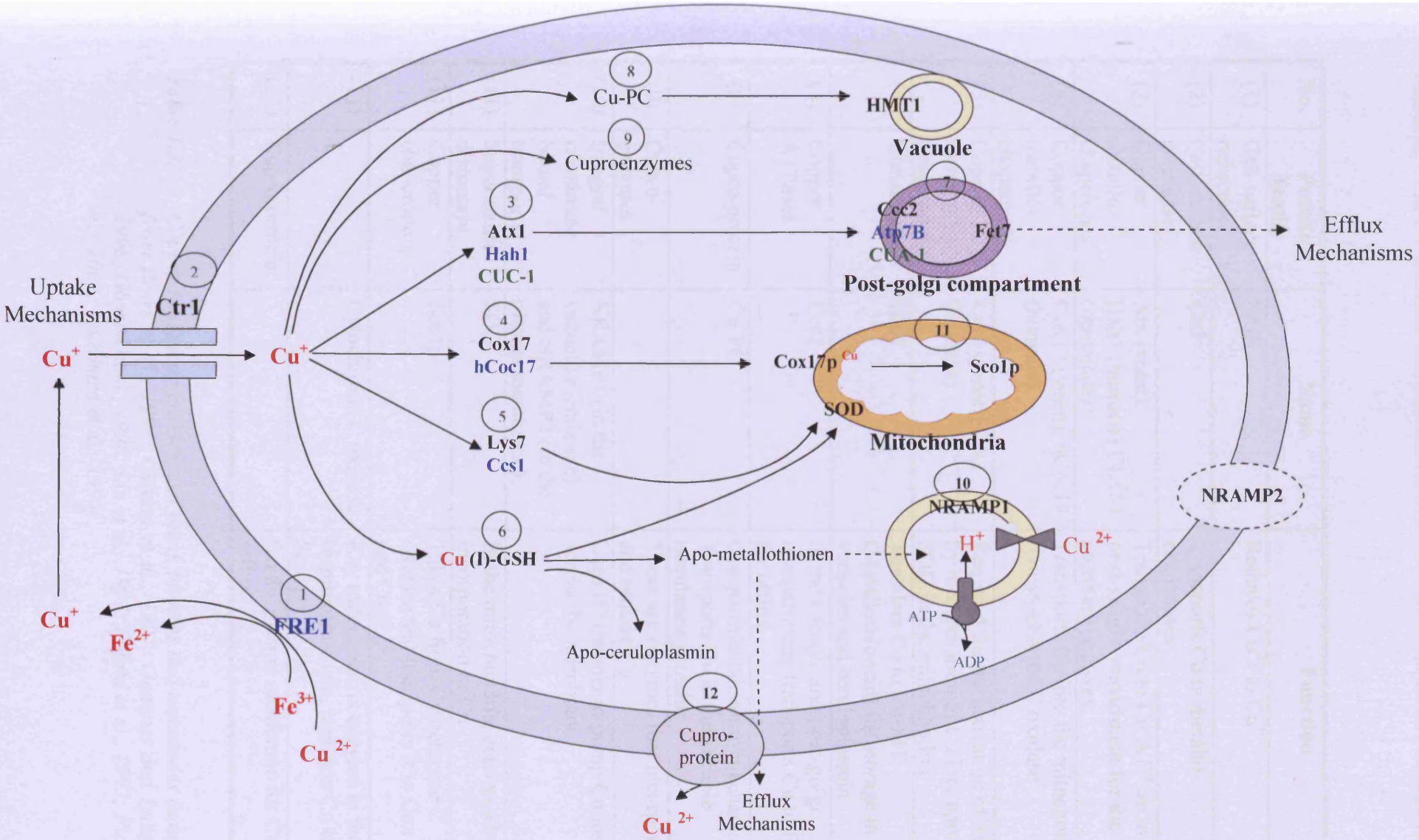

Figure 1.4 *Copper trafficking pathways in yeast (**Black**), humans (**Blue**) and nematodes (**Green**)*

No.	Protein family	Name	Function
(1)	Cell surface reductase	FRE1	Reduces Cu^{2+} to Cu^{+}
(2)	Cell surface permease	Ctr1	Transports Cu to metallo-chaperones
(3)	Copper metallo-chaperones	Atx (yeast), Hah1 (humans) CUC-1 (nematodes)	Transports Cu to Cu-ATPase in the post-golgi compartment for the secretary pathway
(4)	Copper metallo-chaperones	Cox17 (yeast), hCoc17 (humans)	Transports Cu into the mitochondria for cytochrome C oxidase
(5)	Copper metallo-chaperones	Lys7 (yeast), Ccs1 (humans)	Required for the activation of SOD by incorporation of Cu into apo-SOD in the mitochondria
(6)	Glutathiones	GSH	Transfers Cu to Cu-MT (Metallothionein) for storage in vacuoles and ceruloplasmin
(7)	Copper ATPases	Ccc2	In the vacuole and post-golgi compartment, transports Cu to Fet7 for efflux
(8)	Cupro-protein	Cu-PC	Compartmentalisation mechanisms. Transports Cu to the vacuole membrane proteins
(9)	Cupro-enzymes		These are enzymes that incorporate and require Cu
(10)	Integral membrane bound transporter	NRAMP1 (in the vacuole membrane) and NRAMP2 (in the plasma membrane)	Uses H^{+} transfer to pump Cu ions across the membrane
(11)	Super oxide dismutase	SOD	In the mitochondria, activated by incorporation of Cu
(11)	Copper chaperone	Sco1p	Takes Cu from Cytochrome C oxidase and transports it to Cox1 and Cox2
(11)		Cytochrome C oxidase	Key mitochondria enzyme in the respiratory chain, transfers Cu to Sco1p
(12)	Cupro-protein		On the plasma membrane for Cu efflux

Table 1.2. *Copper trafficking pathways in yeast, humans and nematodes (adapted from Beers* et al., *1997; Culotta* et al., *1997; Ganonet and Lauquin, 1998; Glerum* et al., *1996; Lin* et al., *1997; Pufahl* et al., *1997; Puig* et al., *2002; Tsukihara* et al., *1995).*

1.8.2 Ctr protein family

As Cu is necessary for enzymatic functions, uptake mechanisms exist that allow for the entrance of Cu ions into the cell (Nies and Silver, 1995). Cu transport has been most extensively studied in *S. cerevisiae* and led to the discovery of the Ctr family of Cu uptake proteins. Initial uptake of the Cu into cells occurs through the Ctr family of proteins, which function in organisms as diverse as yeast, plants and metazoans including mice and humans. The Ctr family consists of integral proteins that are located on the plasma membrane, as well as on intracellular vacuoler membranes (***Figure 1.4***).

Cu is transported into the cell by two independent and redundant high affinity Cu transporter proteins, Ctr1 (Dancis *et al.*, 1994) and Ctr3 (Knight *et al.*, 1996). Ctr1 is located in the plasma membrane of cells and appears to be ubiquitously expressed in mammalian tissues (Klomp et al., 2002). Ctr1 takes Cu^{2+} from the extracellular space and delivers Cu^{+} into the cell by the action of a cell surface metalloreductase (Opazo *et al.*, 2003). Under conditions of low extracellular Cu concentrations, Ctr1 transcription is activated and turnover appears stable, but high Cu concentrations trigger internalisation and degradation (Dancis et al., 1994). Yeast Ctr2 has recently been localised to the vacuolar membrane, suggesting a potential role in Cu export from the vacuole to the cytoplasm, providing Cu to intracellular chaperones (Rees *et al.*, 2004). ***Table 1.3*** below lists the different Ctr family members and their function.

Name	Function	Location
Ctr1	High-affinity Cu transporter (binds four Cu^{+} ions) which is responsible for Cu uptake in low environmental Cu conditions	Forms complexes with two or three copies at the plasma membrane
Ctr2	Non-essential. Can export stored Cu to the cytoplasm	Assembles as a homo-multimer on the vacuolar membrane
Ctr3	High-affinity Cu(I) transporter responsible for Cu uptake in low environmental Cu conditions	Forms a homo-trimer on the plasma membrane
Ctr4/Ctr5	Cu uptake transporter which resembles a fusion of domains from both Ctr1 and Ctr3	Hetero-dimer on the plasma membrane
Ctr6	Cu release protein	Vacuolar, trimeric

Table 1.3 *The Ctr Cu uptake protein family and their functions*

With the exception of yeast Ctr3, all Ctr family members are rich in methionine residues within the amino-terminal hydrophilic portion of the protein (Labbe *et al.*, 1999). It has been suggested that these residues, arranged as M*XX*M or M*X*M (Mets motifs), are involved in extracellular Cu binding (Dancis *et al.*, 1994). To facilitate translocation of Cu through the membrane channel and further delivery to intracellular Cu chaperones en route to cuproproteins (Puig *et al.*, 2002).

1.8.3 Cut protein family

On the basis of the preliminary characterisation of Cu-sensitive mutants in *E. coli*, it was proposed that 6 genes (cutA, cutB, cutC, cutD, cutE and cutF) are involved in the uptake, intracellular storage and delivery and efflux of Cu. A mutation in one or more of these genes results in increased Cu sensitivity (Gupta *et al.*, 1995). Genetic analyses of the Cu-sensitive *E. coli* mutants and their Cu import and export phenotypes suggested a model for the role of each protein (***Figure 1.5***).

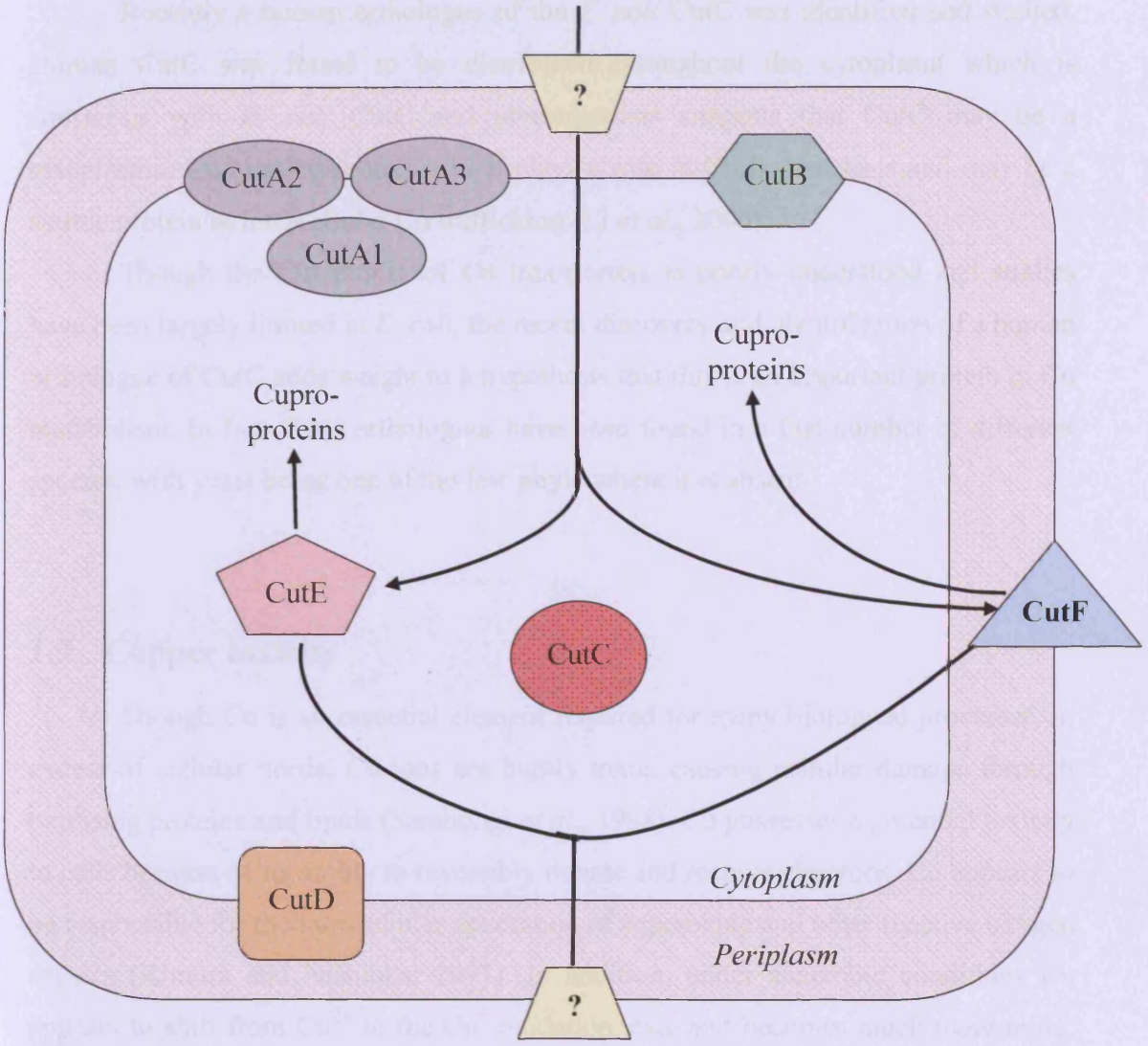

Figure 1.5 *Postulated intracellular Cu transport in* E. coli. *CutA1-3 and CutB cooperate in Cu import across the inner membrane. Containing two different operons, the CutA gene encodes a cytoplasmic protein (CutA1) and two inner membrane proteins (CutA2 and CutA3). CutA2 is a protein disulfide isomerase required for the biogenesis of c-type cytochrome (Odermatt and Solioz, 1995), whilst the functions of CutA1 and CutA3 are not clear (Fong* et al., *1995). CutE has been identified as an intracellular Cu-binding protein (Rogers* et al, *1991) and functions as an apolipoprotein N-acyltranseferase (Gupta* et al., *1997) involved in the modification of one or more minor lipoproteins required for Cu tolerance and cell survival (Gupta* et al., *1993). CutF is an outer membrane lipoprotein and is involved in Cu tolerance in* E. coli *(Gupta* et al., *1995; Snyder* et al., *1995). It is proposed to be responsible for protecting the cell from Cu toxicity and for delivering Cu to sites of assembly of Cupro-proteins. CutC is a cytosolic Cu binding protein and may function as a intracellular shuttle protein in Cu trafficking (Li et al., 2005). CutD may be involved in energy-dependant Cu export (Vulpe and Packman, 1995).*

Recently a human orthologue of the *E. coli* CutC was identified and studied. Human CutC was found to be distributed throughout the cytoplasm which is consensus with *E. coli* CutC and investigations suggests that CutC may be a cytoplasmic Cu-binding protein which plays a role in Cu homeostasis and may be a shuttle protein in intracellular Cu trafficking (Li *et al.*, 2005).

Though the Cut family of Cu transporters is poorly understood and studies have been largely limited in *E. coli*, the recent discovery and identification of a human orthologue of CutC adds weight to a hypothesis that this is an important protein in Cu metabolism. In fact CutC orthologues have been found in a fast number of different species, with yeast being one of the few phyla where it is absent.

1.9 Copper toxicity

Though Cu is an essential element required for many biological processes, in excess of cellular needs, Cu ions are highly toxic, causing cellular damage through oxidising proteins and lipids (Sambongi *et al.*, 1998). Cu possesses a potential toxicity to cells because of its ability to reversibly donate and receive electrons. Cu appears to be responsible for the intracellular generation of superoxide and other reactive oxygen species (Kimura and Nishioka, 1997). In addition, under anaerobic conditions Cu appears to shift from Cu^{2+} to the Cu^{+} oxidation state and becomes much more toxic; possibly because Cu^{+} can diffuse through the cytoplasmic membrane (Outten *et al.*, 2001).

If allowed to engage in uncontrolled redox chemistry in a cell, Cu can cause devastating and irreparable damage to proteins, lipids and DNA (Rees and Thiele, 2004). It is therefore important that organisms have appropriate mechanisms for uptake and detoxification, as well as possessing cellular sensors to ensure that Cu is present in the cell to drive the essential biochemical processes while preventing its accumulation to toxic levels (Pena *et al.*, 1998).

When an organism comes into contact with a heavy metal such as Cu, it can react in one of three ways. It can exclude the metals or pump them out, ensuring that the internal concentrations never reach harmful levels. Alternatively, they may bind Cu to particular proteins (metalloproteins) or enzymes (cuproenzymes), reducing the concentration of free heavy metal ions available in the cell (Vallee and Ulmer, 1972).

A third possibility is that the metals are localised in specific intra-cellular compartments, thus reducing the exposure levels to metabolic pathways.

It is critical for cells to maintain homeostatic concentrations of Cu, since abnormally high or low levels can lead to pathological conditions (Mattie and Freedman, 2004). Consuming Cu-contaminated water or foods is associated with development of acute gastrointestinal symptoms (Knobeloch *et al.*, 1998; Spitalny *et al.*, 1984). Cu poisoning may result in weakness, lethargy and anorexia in the early stages (Semple *et al.*, 1960; Winge and Mehra, 1990) as well as erosion of the epithelial lining of the gastrointestinal tract, hepatocellular necrosis in the liver, and acute tubular necrosis in the kidney (Barceloux, 1999).

Animals vary by species in their sensitivity to Cu intake. Rodents, poultry and pigs can chronically tolerate many times their usual daily intakes of Cu. Whilst sheep and dogs are sensitive to high Cu intakes. In humans the estimated lethal dose of Cu in an untreated adult is 10-20 g (Gaetke and Chow, 2003).

In humans Chronic Cu toxicity primarily affects the liver, because it is the first site of Cu deposition after it enters the blood. Cu toxicity is typically manifested by the development of liver cirrhosis with episodes of haemolysis and damage to renal tubules, the brain and other organs. Symptoms can progress to coma, hepatic necrosis, vascular collapse and death (Winge and Mehra, 1990). Chronic Cu toxicity has also been documented in patients receiving dialysis via Cu tubing (Klein *et al.*, 1972), in workers using pesticides containing Cu (Winge and Mehra, 1990) and in infants maintained for long periods on intravenous total parenteral (Beshgetoor and Hambidge, 1998).

Paradoxically, Cu-deficient animals have exhibited anemia, skeletal defects and degeneration of the nervous system, reproductive failure and other effects. Under normal circumstances, dietary Cu deficiency has not been observed in adults, however, it was observed in malnourished children in Peru, with symptoms including anemia, neutropenia and bone mineralisation (Cordano *et al.*, 1964).

Acquired deficiency of Cu, can be effectively treated by administration of the element (Nriagu, 1979). For many years, early Cu replacement in the body has been proposed as a useful therapy for patients. Cu-histidine administered as a physiological complex is taken up by the brain most efficiently (Strausak *et al.*, 2001).

1.10 Copper disorders in humans

Disease and physical and chemical stresses can disrupt the trafficking of metals. When unchecked, many of these metals are capable of catalysing oxidative damage, directly inhibiting essential activities, disrupting signal transduction pathways and disrupting the folding of nascent proteins. Such deleterious reactions are currently thought to be at the centre of many environmental and inherited diseases of metal metabolism (Finney and O'Halloran, 2003).

1.10.1 Environmental disorders

On a cellular level, the pathological effects associated with the accumulation of excess Cu are consistent with oxidative damage of lipids, proteins and nucleic acids (Mattie and Freedman, 2004). Oxidative damage induced by Cu may also be involved in the pathogenesis of neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, familial amyotrophic lateral sclerosis and prion disease (Llanos and Mercer, 2002; Sharp, 2003). This family of prion diseases includes Creutzfeld-Jacob disease (CJD), Kuru, Gerstmann-Straussler-Scheineker (GSS) disease and fatal familial insomnia (Prusiner, 1991).

Neuronal damage caused by stroke and ischemia (lack of oxygenated blood flow to tissues or organs) may also be associated with pathological disruption of Cu trafficking. It remains to be seen whether the excessive metal accumulation, aberrant protein folding, and/or extensive oxidative damage that are apparent at relevant sites in the brain and nervous system of afflicted individuals are the initiating events, protective response or end products of the specific disease (Finney and O'Halloran, 2003).

Also, Cu metabolism is altered in inflammation, infection and cancer. In contrast to iron levels that decline in serum in infection and inflammation, Cu concentrations and ceruloplasmin rise. Cu itself is important for immune responses including the production of IL-2 by activated lyphocytic cells and supports the activity and effectiveness of cellular and hormonal immunity (Tapiero *et al.*, 2003). Interestingly, most of the disease-causing mutations found to date have been in proteins involved in export of metals from the cells (***Table 1.4***).

COPPER-CONTAINING PROTEIN	KNOWN OR EXPECTED LOSS OF FUNCTION PHENOTYPE
Ceruloplasmin	Cellular ion retention in liver and central nervous system, leading to neurodegenerative disease, iron deficiency anemia
Cytochrome oxidase	Metabolic acidosis, liver failure, encephalopathy, hypertrophic cardiomyopathy
Dopamine beta hydroxylase	Increased embryonic lethality , cold intolerance
Factor V, factor VII	Bleeding tendency
Hephaestin	Iron deficiency anemia with stainable iron in intestinal epithelial cells
Lysyl oxidase	Loss of skin elasticity, hyperextensibility, vascular anomalies/ aneurysms
Cu/Zn SOD	None without further manipulations

Table 1.4 *Cu-containing proteins and expected/known defects in their absence* (Andrews, 2001).

1.10.2 Inherited genetic disorders

The discovery of the genetic basis of Menkes and Wilson diseases, began to focus a new spotlight on intracellular metal ion metabolism (Andrews, 2001). These two inherited genetic disorders are caused by mutations of two closely related Cu transporting P-type ATPases. These P-type ATPases which form a family of more than 70 members in bacteria, fungi, plants and animals, function as monomers to pump cations through membranes using ATP (Vulpe and Packman, 1995).

Mutations in these P-type ATPases, ATP7A and ATP7B lead to disorders of Cu starvation (Menkes Disease) and Cu toxicity (Wilson Disease), respectively (Sambongi *et al.*, 1998). Both Menkes disease and Wilson disease can result in defects of a secreted protein (lysyl oxidase and ceruloplasmin, respectively) as well as in defects of Cu transport into lysosomes.

The clinical manifestations of Menkes disease can be ascribed to reduced activity of several Cu requiring enzymes (Harrison and Dameron, 1999). While in the early stages of Wilson disease, Cu accumulates diffusely in the cytoplasm bound to MT. Then later is sequestered as Cu-associated proteins in lysosomes (Vulpe and Packman, 1995).

Another disorder leading to Cu toxicity called Idiopathic Cu toxicosis, or non-Indian childhood cirrhosis is similar to Wilson's disease and involves a rare inherited genetic defect in Cu metabolism (Muller *et al.*, 1998). As well as its genetic inheritance it is thought to be caused by an environmental factor. In Indian households the use of Cu cooking utensils and the high Cu levels in milk boiled in such vessels have led some investigators to suggest an environmental etiology for the disorder. However, autosomal recessive inheritance patterns have also been indicated (Vulpe and Packman, 1995). ***Table 1.5*** summaries the inherited genetic Cu disorders in humans.

DISEASE	PROTEIN AND LOCATION	NORMAL PROTEIN FUNCTION	LOSS OF FUNCTION	CLINICAL MANIFESTATIONS
Menkes disease (X-linked recessive disease occurring in 1 in 200,000 live births	MNK (ATP7A) Expressed in many cell types and predominantly localised to the trans-Golgi network	When cells are exposed to excessive Cu, MNK relocates to the plasma membrane where it functions to pump Cu out of intestinal and renal epithelia cells	Cu deficiency results from an inability to mobilise absorbed Cu from intestinal epithelial cells	Leads to mental retardation, neurological degeneration, steely grey hair, aortic aneurysms, loose skin, bony abnormalities and death in infancy
Wilson disease (autosomal recessive)	WD (ATP7B) Trans-membrane Cu exporter which is highly expressed in the liver	Essential for the excretion of cerulo-plasmin Cu from hepatic lysosomes into bile and may be responsible for the maintenance of the normal zero balance of Cu	Cu overload due to loss of the ability to export Cu from the liver to the bile and the inability to incorporate Cu into ceru-loplasmin. Accumulation of Cu in the brain, kidneys and liver unless dietary intake is controlled	Cu build up in the liver leads to cirrhosis and is deposited in other tissues, including the brain and cornea of the eye; causing basal ganglia abnormalities, cerebral atrophy, movement disorders, psychiatric disturbances and dementia. Patients die either due to progressive liver failure or neurological deterioration, unless they are treated with chelating agents or zinc to promote the excretion of Cu
Indian childhood cirrhosis (ICC). Environ-mental and genetic	Unknown	NA	Children with ICC accumulate massive amounts of Cu in the liver	Patients develop cirrhosis and liver failure and until recently invariably died in infancy. Now the disorder can be treated with D-penicllamine

Table 1.5 *Properties of inherited Cu disorders in Humans (Andrews, 2001; Gaetke and Chow, 2003; Harrison and Dameron, 1999; Nriagu, 1979; Strausak* et al., *2001; Tapiero* et al., *2003; Vulpe and Packman, 1995).*

These disorders which result in both Cu overload and Cu deficiency demonstrate the essential yet toxic nature of Cu. Cu homeostasis requires the ability of the Cu ion sensors to detect Cu and respond by appropriately regulating the expression of Cu homeostasis genes in order to maintain the delicate balance between essential and toxic levels (Pena *et al.*, 1998).

1.11 Free radicals

Several mechanisms have been proposed to explain Cu-induced cellular toxicity. Most often, the basis for these theories is the propensity of the Cu ions to participate in the formation of free radicals such as reactive oxygen species (ROS) (Bremner, 1998; Kadiiska *et al.*, 1993). A free radical is any chemical species (atom, ion or molecule) that contains an unpaired or odd number of electrons. Although normally bound to proteins, both cupric and cuprous Cu ions may be released and become free to catalyse the formation of highly reactive free radicals through oxidation and reduction reactions (Gaetke and Chow, 2003). In the presence of superoxide ($*O_2$-) or reducing agents such as ascorbic acid or GSH, Cu^{2+} can be reduced to Cu^{+}, which is capable of catalysing the formation of hydroxyl radicals (OH*) from hydrogen peroxide (H_2O_2) via the Haber-Weiss reaction (Bremner, 1998; Kadiiska *et al.*, 1993).

$$*O_2^- + Cu^{2+} \rightarrow O_2 + Cu^{+}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\bullet}$$

The hydroxyl radical is the most powerful oxidising radical likely to arise in biological systems, and is capable or reacting with practically every biological molecule (Buettner, 1993). It can initiate oxidative damage by abstracting the hydrogen from an amino-bearing carbon and from an unsaturated fatty acid to form a lipid radical (Powell, 2000). These lipid peroxides decompose to yield a cascade of reactions including the formation of the known mutagen malondialdehyde (Tapiero *et al.*, 2003). Lipid peroxides have been shown to produce an irreversible impairment of membrane fluidity and elasticity which can lead to the rupture of cells. Free radical

activity has also been shown to oxidise and cross-link proteins including enzymes and connective tissue. The reaction of an oxygen radical with DNA can knock out a base or cause a strand breakage, with the potential to produce harmful or lethal events (Tapiero *et al.*, 2003).

Data obtained from *in vitro* and cell culture studies are largely supportive of Cu's capacity to initiate oxidative damage and interfere with important cellular events. Oxidative damage has been linked to chronic Cu overload and/or exposure to excess Cu caused by accidents, occupational hazards and environmental contamination. Additionally, Cu-induced oxidative damage has been implicated in disorders associated with abnormal Cu metabolism and neurodegenerative changes (Gaetke and Chow, 2003). In addition, Cu can be toxic by directly binding to sulfhydryl groups in proteins, which results in enzyme inactivation or altered protein conformation (Mattie and Freedman, 2004).

Interestingly, a deficiency in dietary Cu also increases cellular susceptibility to oxidative damage as protection from these oxidative stresses is inhibited. These defences include SOD, catalase and glutathione peroxide which detoxify superoxide radicals, hydrogen peroxide and lipid hydroperoxides respectively (Gaetke and Chow, 2003).

1.12 The aging theory

Most organisms undergo a natural aging process over their finite lifespan. Research on the aging process has promoted many theories of the biological how and why of aging, such as the free radical theory of aging. This theory postulates that the ROS generated as a by-product of energy generation or other metabolic reactions accumulate over time, causing damage to cellular components and leading to a reduction in mitochondrial function and eventually cell death (Rees and Thiele, 2004).

The idea that aging is a result of free radical damage is often credited to Denham Harman who in 1956 based his theory on the observation that irradiation of living things known to induce the formations of free radicals shortened their life span and produced changes that resembled aging (Wickens, 2001).

The free radical theory may also be used to explain many of the structural features that develop with aging including the lipid peroxidation of membranes, formation of age pigments, cross-linkage of proteins, DNA damage and decline of

mitochondrial function (Wickens, 2001). In order to investigate the role of Cu toxicity on cellular processes such as aging and development, model organisms can provide a good platform for research.

1.13 Model organisms

To facilitate the understanding of heavy metal toxicity, its effects at the molecular level and its relationship with human diseases, model organisms form a key basis to work from. Many aspects of biology are similar in most or all organisms and model organisms have strong advantages for experimental research. A model system is simple, readily accessible and easily manipulated. When selecting living organisms as models to work with, certain criteria are used depending upon the experimental purposes. As a result, there is a wide range of characteristics common to model organisms, including: 1) rapid development with short life cycles, 2) small adult size, 3) availability and 4) tractability (Bolker, 1995).

Several vertebrate and invertebrate models are currently utilised to study biological and genetic responses. Rats and mice are used for mammalian models but do not offer the advantages and ease of using "lower" organisms. Popular lower organisms include bacterium (*Escherichia coli*) and yeast (*Saccharomyces cerevisia*). The fruit fly (*Drosophila melanogaster*) and the nematode (*Ceanorhabitis. elegans)* represent invertebrate model organisms. *C. elegans* are a popular research organism as they possess all the characteristics mentioned, yet share many essential biological properties with humans.

1.13.1 Nematodes (a model organism)

Nematodes (roundworms) are amongst the most abundant organisms on the earth and reside in a range of habitats which are unsurpassed by any other metazoan group. Many nematodes are parasitic in animals including man and domestic animals.

Important early work on the nematode model was done by E. C. Dougherty, V. Nigon and their respective colleagues between 1945 and 1965. Then in 1965, Sydney Brenner chose the free-living nematode *Ceanorhabiti elegans* as a promising model for a concerted genetic, ultrastructure and behavioural investigation of development and function in a simple nervous system (Wood, 1988). The recent surge

of interest, however, is due largely to the detailed studies on the genetics and anatomy of *C. elegans*, which began in Sidney Brenner's laboratory about 1974 (Zuckerman, 1980). The resultant effort has brought together geneticists, cell and developmental biologists, neurologists, behavioralists, endocrinologists, toxicologists, nutritionalists and gerontologists; all with a common interest in focusing on the nematode as a biological model in order to examine some of the most basic processes of life.

To date *C. elegans* is the best characterised multicellular eukaryote, not only because its entire genome is known, but also because the ecology, life-cycle, morphology and cellular development and function of each cell lineage has been mapped in exquisite detail and described in full. *C. elegans* live in the soil and rotting vegetation throughout the world, where they survive by feeding on microbes. They are built from two tubes one inside the other; the outer tube is a tough skin or cuticle, whilst the inner tube contains the mouth, digestive tube, sex organs and anus (***Figure 1.6***).

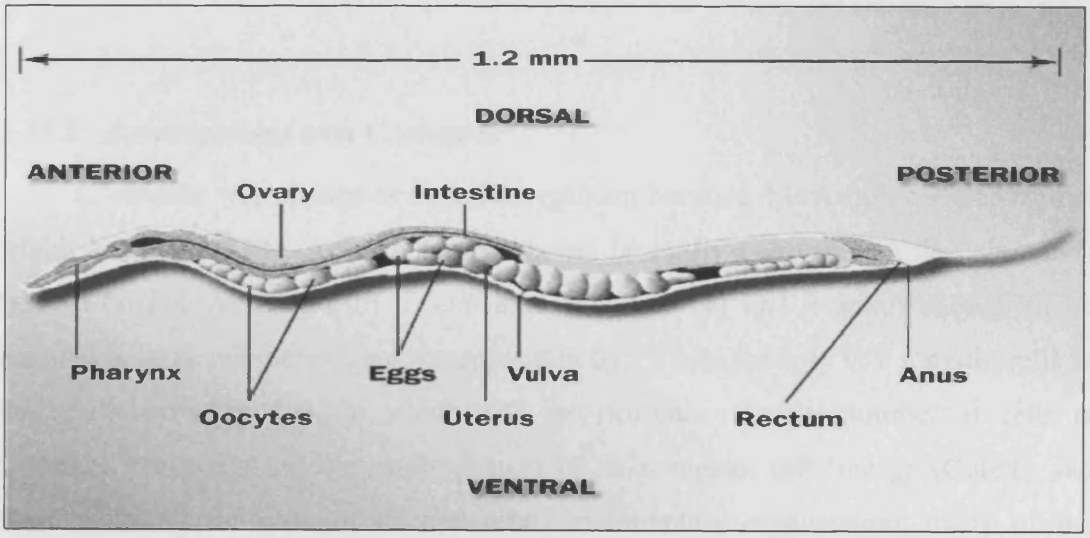

Figure 1.6 *Diagram of a hermaphrodite* C. elegans *anatomy (taken from http://www.imsc.res.in/~sitabhra/research/neural/celegans/index.html, 2006).*

There are two sexes, a self fertilizing hermaphrodite and a male. As the species is largely hermaphrodite mutant populations may be easily cloned from a single adult. Homogeneity of the genotype is a critical prerequisite for population studies of genetic inheritance (Croll and Matthews, 1997). In wild type *C. elegans* males are found at about 0.05% of the population. They develop after accidental non-disjunction of the X chromosomes in gametogenesis, making them XO (Herman *et al.*, 1982). The development and function of this diploid organism is encoded by an estimated 21,000 distinct genes and the 97 million base pair sequence of the *C. elegans* genome is now complete (Sanger Centre and Genome Sequencing Centre).

C. elegans offers multiple advantages over other genetic model organisms as they can be grown and manipulated with the speed and ease of micro-organisms, while offering the features of a higher organism such as epithelia, intestine, muscle and a complex sensory system. Furthermore most genes and pathways shown to be important in cell, developmental and disease biology are conserved between nematode and man. Therefore, the nematode is an excellent model of biological systems in general, and potentially a powerful tool to unravel human disease.

1.13.2 Investigations with C. elegans

C. elegans was chosen as a model organism because it is a multicellular animal which has a short life cycle (2-3 weeks), can be easily cultivated in the laboratory (grown on Petri dishes, with *E. coli* as a food source) and is small enough to be handled in large numbers (1mm transparent body). There are only 959 somatic cells in the adult hermaphrodite, of which 302 are neurones. As the number of cells is invariant it has enabled the establishment of its complete cell lineage (Culetto and Sattelle, 2000). In spite of its apparently rudimentary organisation, many of the distinct cell types associated with complex functions in mammals such as muscle cells, neurones, gut and excretory cells can be recognised and identified uniquely in *C. elegans* (Culetto and Sattelle, 2000). Therefore *C. elegans* has many of the essential biological characteristics that are central to problems of human biology (embryogenesis, morphogenesis, development, nerve function, behaviour, aging etc).

The generation time of *C. elegans* is rapid and takes about 3 days (20°C). The eggs are fertilized upon passing the spermatheca and a tough chitinous shell forms

around the egg. The eggs start to cleave inside the mother and are laid at about the 30-cell stage. Embryogenesis is very regular, and a juvenile with about 550 cells hatches from the egg case. In the period between hatching and adult stage, the animal feeds and grows not only in size, but also in cell number, passing through four larval stages L1 to L4, and four molts (***Figure 1.7***). Egg laying begins about 50 h after hatching and each hermaphrodite produces 200-300 progeny (Zuckerman, 1980).

C. elegans are also able to go into dauer stage where they may survive for about 3 months without food. If food becomes scarce due to a high population density, *C. elegans* secretes a pheromone that causes the worms to arrest development until conditions improve. In this dauer stage the larvae are considered to be non-aging because the period spent in this alternate phase has no effect on the lifespan of the worm once it goes back to normal development (Riddle *et al.*, 1997).

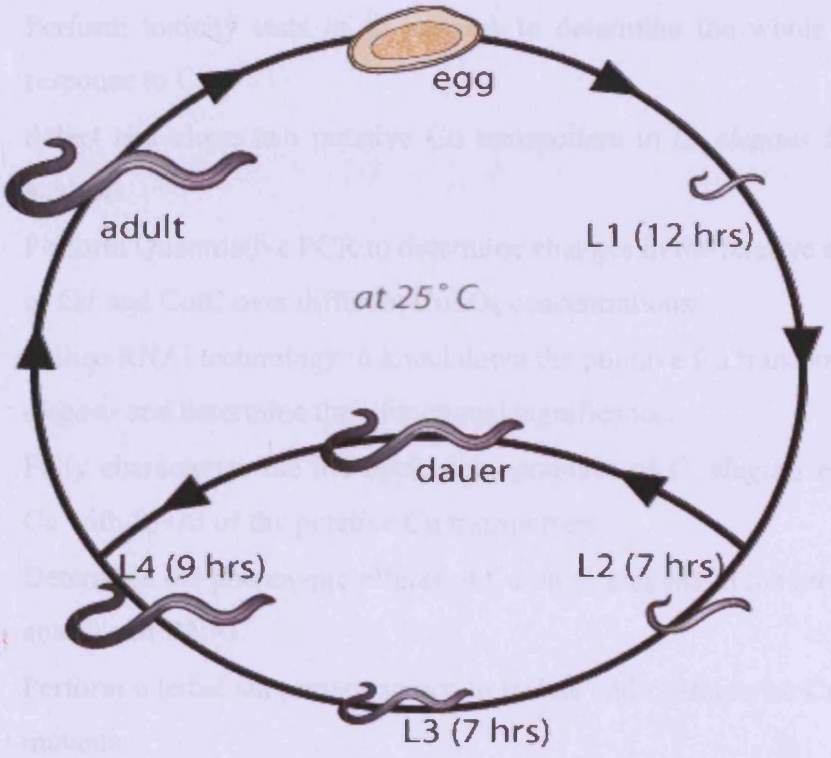

Figure 1.7 *Schematic of* C. elegans *life cycle. The lifecycle is slowed at lower temperatures and can be interrupted by the dauer stage if environmental conditions are not favourable (taken from www.bioteach.ubc.ca/MolecularBiology/ Longevity//, 2006)*

1.14 Aims and objectives

Environmental pollution impacts on an organism's utilisation of its genome and this results in consequences for the organism's biology thus ultimately affecting population dynamics. It is therefore important to assess the effects of Cu toxicity on the ecosystem and its natural communities. The overall aim of this project was to investigate Cu homeostasis and toxicity using the soil dwelling nematode, *Caenorhabitis elegans* as a model organism. Therefore the objectives of this project were to investigate the whole organism response to Cu pollution in *C. elegans* and to determine the functional significance and expression pattern of two putative Cu transporters Ctr and CutC.

The objectives were to:

- Perform toxicity tests in *C. elegans* to determine the whole organism response to Cu.
- Select and clone two putative Cu transporters in *C. elegans* for further analysis.
- Perform Quantitative PCR to determine changes in the relative expression of Ctr and CutC over different $CuSO_4$ concentrations.
- Utilise RNAi technology to knockdown the putative Cu transporters in *C. elegans* and determine their functional significance.
- Fully characterise the life cycle demographics of *C. elagans* exposed to Cu with RNAi of the putative Cu transporters.
- Determine the phenotypic effects of Cu on *C. elegans* in the presence and absence of RNAi.
- Perform a lethal suppressor screen to isolate and characterise Cu resistant mutants.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 Materials

The sources of materials, reagents and buffers routinely used in this study are summarised in ***Table 2.1***. Reagents were of molecular biology grade, unless otherwise stated. Specific solutions were prepared according to the compositions listed in ***Table 2.2.***

SUPPLIER	REAGENT
Applied Biosystems (Foster City, CA, U.S.A)	Big Dye terminator V3.0 Cycle Sequencing kit
BDH Laboratory Supplies (Dorset, UK)	Copper sulphate and Sodium dodecyl sulphate (SDS)
Bioline (London, UK)	Agarose, NZCYM agar capsules, NZCYM broth capsules and SOC media capsules
Calbiochem (Nottingham, UK)	Tween 20
Clontech (Oxford, UK)	Advantage HF2 *Taq* polymerase
DIFCO Laboratories (MD, USA)	Agar technical and Bactopeptone
Eurogentec (Southampton, UK)	Custom synthesised TaqMan® probes
Fisher Scientific (Leicestershire, UK)	Chloroform, ethylenediaminetetracetic acid (EDTA), Ethanol, Glucose, Glycerol, HEPES, *Iso*-propanol, Magnesium Chloride, Magnesium sulphate, Potassium Chloride, Potassium di-hydrogen orthophosphate (anhydrous), Potassium hydroxide, Sodium acetate, Sodium bicarbonate, Sodium chloride, Sodium hydroxide and Sodium hydroxylamine
Invitrogen Ltd. (Paisley, UK)	DH5α cells
Life Technologies (Paisley, UK)	10 X TAE
MWG (Milton Keynes, UK)	Custom synthesised primers
Promega Corporation (Southampton, UK)	2X rapid ligation buffer, 10X digest buffer, BSA, Calf intestinal alkaline phosphatase (CIAP), CIAP 10X buffer, dNTPs, Klenow DNA polymerase I, Moloney murine leukemia virus reverse transcriptase (M-MLV RT), Molecular weight markers, *Pfu* DNA polymerase, pGEM-T vector, Restriction enzymes Ribonuclease H, Ribonuclease (RNAse) A, Ribonuclease inhibitor (RNAsin), T4 DNA ligase, *Thermus aquaticus (Taq)* buffer, *Taq* polymerase, Wizard® Plus SV Miniprep DNA Purification Kit, Wizard® Plus SV Maxiprep DNA Purification Kit and X-gal
Qiagen (Crawley, UK)	QIAquick Gel extraction kit and RNeasy mini kit
Roche Diagnostics (East Sussex, UK)	Chloramphenicol, Shrimp alkaline phosphatases and Tris (2-amino-2 (hydroxylmethyl)-1,3 propanediol)
Sigma Chemical Company (Dorset, UK)	Ampicillin, Bromophenol blue, β-mercaptoethanol, Calcium Chloride, Cholesterol, Dithiothreitol (DTT), Ethidium bromide, Formaldehyde, High Performance Liquid Chromatography (HPLC) water, Iron sulphate, Isopropyl β-D-thiogalactopyranoside (IPTG), Luria broth (LB) agar tablets, LB broth tablets, Phenol, Methanesulfonic acid ethyl ester (EMS), Nystatin, Sephacryl 300 HR, Sodium hypochlorite solution, Syber Green and Tri-reagent

Table 2.1. *Table of reagents and their supplier*

PROCEDURE	SOLUTION	COMPONENTS
Nematode agar plates	NGM agar	50mM NaCl, 0.25% (w/v) Bactopeptone, 1.7% (w/v) Agar Technical. Autoclave then add 1mM $CaCl_2$, 1mM $MgSO_4$, 25mM K_2HPO_4 (pH6), 0.1% (v/v) Cholesterol stock and Nystatin (1U/ml) and mix well
	Cholesterol	5% (w/v) Cholesterol dissolved in Ethanol
Agarose gel electro-phoresis	Electrophoresis loading dye	50 % (v/v) Glycerol, 1 % (w/v) Bromophenol blue and 1x TAE (sterilised)
	10x TAE	40mM Tris-acetate and 1mM Na_2-EDTA (pH 7.6)
Egg preparation	Bleach mix	12% (v/v) NaOCl (4%) and 0.25M KOH
	M9 Buffer	22mM KH_2PO_4, 42mM Na_2HPO_4, 85.5mM NaCl and 1mM $MgSO_4$
Freezing nematodes	Freezing solution	100mM NaCl, 50mM KPO_4 (pH6) and 30% (v/v) Glycerol. Autoclave then add 0.3mM sterile $MgSO_4$
Genomic DNA extraction	Genomic lysis buffer	0.2M NaCl, 0.1M Tris (pH 8.5), 50mM EDTA and 0.5% (v/v) SDS
Liquid culture for nematodes	S-Basal media	0.1M NaCl, 5.7mM K_2HPO_4 and 44mM KH_2PO_4. Autoclave then add 0.1% (v/v) Cholesterol stock
Polymerase chain reaction	10x *Taq* polymerase buffer	High-throughput PCR buffer and 0.1 % Triton®X-100
Single worm PCR	Worm lysis buffer	25mM KCl, 25mM Tris (pH 8.2), 1.25mM $MgCl_2$, 0.1% (v/v) NP40, 0.1% (v/v) Tween-20 and 0.005% (v/v) Gelatin. Add 25% (w/v) Proteinase K prior to use
TaqMan quantitative PCR	10X buffer	2M Tris-HCl (pH8.3), 2M KCl, 1M $MgCl_2$ and 100µM ROX
***Taq* polymerase production**	Buffer A	50mM Tris-HCl (pH8, 50mM d-Glucose and 1mM EDTA
	Pre-lysis buffer	Buffer A plus Lysozyme (4mg/ml).
	Lysis buffer	10mM Tris_HCl (pH8), 50mM KCl, 1mM EDTA, 1mM PMSF, 0.5% (v/v) Tween-20 and 0.5% (v/v) Nonidet-P40
	Storage buffer	50mM Tris-HCl (pH 8.0), 50mM KCl, 1mM DTT, 0.5mM PMSF and 50% (v/v) Glycerol
Wizard® Plus SV miniprep kit (Promega)	Cell resuspension solution	50mM Tris (pH 7.5), 10mM EDTA and 100µg/ml RNase A
	Cell lysis solution	0.2M NaOH and1% SDS
	Neutralization solution	4.09M Guanidine Hydrochloride, 0.76M Potassium Acetate and 2.12M Glacial Acetic Acid (pH 4.2)
	Column wash	60mM Potassium Acetate, 10mM Tris-HCl (pH7.5) and 60 % Ethanol

Table 2.2 *List of reagents and buffers and their compositions.*

2.1.1 Preparation of solutions, glassware and plasticware for DNA/RNA investigations

Two distinct grades of water were used, namely double distilled water (ddH_2O) or filtered HPLC grade water (for all applications involving DNA and RNA manipulations). All glassware (beakers, bottles, flasks, etc.) and plasticware (pipette tips, 1.5ml tubes etc) were sterilised before experiments by autoclaving at a pressure of 975kPa at 120°C for 20min. All solutions used in RNA and DNA studies were also autoclaved under the same conditions. When handling RNA, pipette tips with filters were always used to stop aerosol contamination of samples.

For increased protection against ribonuclease (RNase) contamination, containers were autoclaved twice for RNA work. Heat sensitive solutions (e.g. antibiotics) were sterilised using a syringe and pushed through a sterile 0.22μm filter (Millex) into an RNase free, sterile container.

2.1.2 Media

Luria Broth (LB) media and LB agar were prepared with ddH_2O from tablets according to the manufacturer's instructions and autoclaved prior to use (***Section 2.1.1***). SOC was prepared by the addition of glucose (final concentration of 20mM) to autoclaved SOB. Following sterilisation, the medium was left to cool to below 50°C and where required, antibiotics were added at an appropriate concentration as described in ***Section 2.1.3.***

2.1.3 Antibiotics

Aliquots of antibiotic and fungicide stocks were prepared to the desired concentration with dH_2O, ethanol or DMSO and then filter-sterilised through 0.22μm filters (Nucleopre™, Millex, Millipore) into 1.5ml tubes and stored at -20°C (***Table 2.3***). Antibiotics were then added to solutions at temperatures below 60°C.

Antibiotic/ fungicide	Abbreviations	Stock concentration (mg/ml)	Final concentration (μg/ml)
Ampicillin	Amp	100^{H}	100
Chloramphenicol	Cm	50^{E}	30
Kanamycin	Kan	100^{H}	50
Tetracycline	Tet	50^{D}	5
Nystatin		10^{H}	1

Table 2.3 Preparation of antibiotic stocks. ^{H}- Prepared in dH_2O. ^{E}- Prepared in ethanol. ^{D}- Prepared in DMSO.

2.1.4 Host strains and vectors

Various host strains of *Escherichia coli (E. coli)* and vectors were required for cloning and expression studies. The *E. coli* strains utilised and their application are listed in ***Table 2.4*** and the vectors used are shown in ***Table 2.5.***

Strain	Genotype	Application	Supplier
DH5α	*F⁻ ϕ80d lacZΔM15 Δ(lacZYA -argF)U169 deoR recA1 endA1 usdR17(r_{K-}, M_{K+}) pho A supE44 λ⁻ thi-1 gyrA96 relA1*	Competent cells used for standard transformations	Invitrogen
HT115	*F-, mcrA, mcrB, IN (rrnD-rrnE)1, lambda-, rnc14::Tn10 (DE3 lysogen:lacUV5 promoter – T7 polymerase*	RNase III deficient with IPTG-inducible T7 polymerase activity allowing expression of dsRNA for RNAi	Fire Lab
OP50		A uracil-requiring mutant of *E. coli* to prevent overgrowth of the bacterial lawn for feeding nematodes	Fire Lab

Table 2.4. *Strains of E. coli used and their application.*

Vector	Feature	Application	Supplier
pGEM-T	A high copy number plasmid (Amp^R) with thymidine base overhangs to prevent re-ligation, and a β-galactosidase gene for blue/ white screening	Non-expression cloning	Promega
ppD129.36	T7 RNA promoter, ensuring low background expression of proteins until induction by IPTG to produce dsRNA (Amp^R)	RNAi	Fire Lab
pTaq vector	Contains *Taq* polymerase	*Taq* polymerase purification	

Table 2.5 *Vectors used and their application.*

2.1.5 DNA Markers

The DNA markers used were lambda DNA digested with *Hin*dIII (λDNA/*Hin*dIII) and ϕX174 digested with *Hae*III (ϕX174/*Hae*III). The fragment sizes of these markers in base pairs (bp) are given in ***Table 2.6*** and shown in ***Figure 7.1***

DNA marker	Fragment sizes (bp)
λ/*Hin*d III (λH3)	23130, 9416, 6557, 4361, 2322, 2027, 564, 125.
ϕX174/*Hae* III (ϕH3)	1353, 1078, 872, 603, 310, 281, 271, 234, 194, 148, 72.

Table 2.6 *DNA marker used and their fragment sizes (bp)*

2.2 *Caenorhabditis elegans* husbandry

The *C. elegans* wild type laboratory strain N2 was used in all experiments unless otherwise stated. *C. elegans* stocks were maintained on NGM agar in 55mm Petri dishes at 20°C, in a constant temperature incubator (Sanyo instruments). Temperatures of 15°C and 25°C were also used to slow down or speed up the *C. elegans* life cycle respectively. The *E. coli* strain OP50 was used as a food source unless otherwise stated.

2.2.1 Preparation of C. elegans *stock plates*

NGM agar (8ml) was dispensed into 55mm triple-vent sterile Petri dishes using an automated plate pourer (Masterfex, Cole-Parmer instrument Compnay) with previously autoclaved plastic dispenser tubes. About 10ml of Luria broth (LB) was inoculated with OP50 from a glycerol stock using a sterile tip. The culture was grown overnight (O/N) at 30°C in a shaking incubator. OP50 (100µl) was spread onto each NGM agar plate and allowed to grow for 1 day at room temperature (RT).

2.2.2 Transferring C. elegans

Two methods were used to transfer *C. elegans* from one agar plate to another. To transfer single worms a "worm pick" was used. A worm pick consists of a glass pasteur pipette with a platinum wire hook at one end. Under a dissecting microscope (Nikon SMZ800) the nematode was carefully lifted from one plate to another. To transfer a large number of nematodes at one time, a chunk of agar was removed from the plate using a scalpel sterilised by an ethanol flame.

2.2.3 Freezing stocks of C. elegans

To maintain stock levels, *C. elegans* strains were stored in aliquots at -80°C. The *C. elegans* used for freezing were washed off agar plates (using 3ml S-Basal media) into a 15ml Falcon tube, 1 day after the bacteria food source was exhausted. Thus, they were predominately L1 but not yet entered into dauer stage. The tubes were centrifuged at 3000g for 1 min. All but 1.5ml of the supernatant was removed and freezing solution (1.5ml) was added. The nematodes were mixed well and pipetted

into 1ml aliquots in 1.5ml Microcentrifuge tubes. The nematodes were frozen slowly to -80°C by placing the tubes between styrofoam racks. To ensure viability, the next day one aliquot was tested by thawing at RT. Nematodes were placed onto an agar plate and checked for live individuals.

2.3 *C. elegans* egg preparation

To synchronise the age of a population of *C. elegans,* egg preparation was performed. Gravid adults were washed off a stock agar plate with M9 (3ml) and pipetted into a 15ml Falcon tube. The nematodes were collected to the bottom of the tube by centrifugation at 2000g for 2 min (at 16°C) and the supernatant was removed. This wash step was repeated with M9 (6ml) and the supernatant was removed. The bleach mix (5ml) was then added and the tube was shaken vigorously for 5 min. This breaks open the nematodes and releases the eggs which are protected from the bleach by a hard chitinous outer layer. The mix was then washed 5 times by centrifugation with M9 (6ml) as described before. Finally fresh M9 (6ml) was added to the nematodes and the tube was left gently rotating (using a Tube Rotator SB, Stuart Scientific) O/N at 20°C. The eggs hatch but the lack of food causes the nematodes to arrest at L1. The synchronous population continues to grow after addition of food.

2.4 Toxicity tests with *C. elegans*

For standard toxicity tests, *C. elegans* were cultured on NGM agar with the bacteria OP50 as a food source. An appropriate amount of the toxin was added both to the agar and the bacteria to ensure the nematodes were fully exposed to the metal. Plates were left to dry for 1 day, then nematodes were placed on the plates with a number of replicates for each test. The experiments, specific details of which are described in relevant results chapters, were performed at 20°C and the nematodes were transferred to fresh agar plates at appropriate intervals. Demographic and phenotypic observations were made throughout the experiments.

2.5 RNA interference (RNAi) in *C. elegans*

Putative Cu transporters Ctr and CutC were cloned into the RNAi vector ppD129.36 (***Section 2.13***). Positive colonies were identified by PCR (***Section 2.13.6***) and then confirmed by sequencing (***Section 2.14***). ppD129.36 constructs containing the gene of interest or the empty ppD129.36 vector as a control were transformed into HT115 competent cells (***Section 2.13.5***). Single colonies were picked and grown in 10ml LB broth plus Amp (LB+Amp) at 37°C with shaking (200rpm) O/N. The culture (100µl/plate) was spread onto NGM agar plates (supplemented with IPTG (1mM), Amp and an appropriate amount of $CuSO_4$). $CuSO_4$ was also added to the bacterial culture before spreading onto the agar, to ensure the nematodes were directly exposed to the Cu. Plates were left to dry for 1 day, then nematodes were placed on the plates with a number of replicates for each test. The experiments, specific details of which are described in ***Table 2.7***, were performed at 20°C and the nematodes were transferred to fresh agar plates at appropriate intervals.

Test	Description	No. of replications	Experimental
Brood Size	Total number of progeny produced	20	Place individual L4s on plates and transfer every 36 hours. Count the number of progeny produced on each plate
Brood Period	Time between first and last egg laid	20	Place individual L4s on plates and transfer every 36 hours. Every 2 hours check for eggs and note the time between first egg and last egg laid
Generation time	Time from egg to egg	30	Place gravid adults on plates and leave for 2 hours to lay eggs. Once eggs reach L4s transfer individuals onto fresh plates until the first eggs are produced
Life Span	Time from egg to death	100	Adults were placed on plates O/N to lay eggs. Eggs were allowed to develop until L4 then 10 worms were pooled per plate, with 10 plates for each test. The time of death was noted

Table 2.7. *Experimental design of life-history analysis in* C. elegans

2.6 Random mutagenesis of *C. elegans*

Egg preparation was performed from six large plates (90mm Petri dish) of nematodes following the methods described in ***Section 2.3***. The eggs were left to hatch at 20°C rotating O/N. To estimate the number of worms present a titre was performed the following day. The nematodes were then dispensed onto 60 large plates and maintained at 20°C until they reached L4 larva. The nematodes were again washed off the plates using M9 into a 15ml conical flask. The volume was reduced to 8ml by centrifugation at 2500g for 2 minutes. The mutagen Methanesulfonic acid ethyl ester (EMS, 160µl) was added to 8ml of OP50 culture, which in turn was then added to the nematodes. The mix was split into four 4ml aliquots and left to rotate at 20°C overnight. The nematodes were washed thoroughly 7 times with M9 (6ml) by centrifugation as described before. The nematodes were then dispensed onto 60 large plates and incubated at 20°C until they reached adulthood.

An egg preparation was performed as before (***Section 2.3***) and a titre was carried out to estimate the number of nematodes present. After washing, the nematodes were transferred to a 1L liquid culture of S-basal media containing $CuSO_4$ (2mM). The nematodes were left shaking (200rpm) at 20°C for 10 days. Titres were performed on a daily basis to monitor numbers of nematodes. Following the exposure period the culture was spun down and washed thoroughly with M9 by centrifugation as described before. Surviving nematodes were placed individually onto NGM agar with OP50 and incubated at 20°C. After two generations when stock levels reached a sufficient level, each mutant was frozen (***Section 2.2.3***) and stored at -80°C for future use.

2.7 Atomic absorption spectrophotometry (AAS)

The concentration of metal ions present within NGM agar was measured by AAS. Agar (1g) was weighed out into flat bottom glass tubes (acid washed). Concentrated nitric acid (1ml) was added to each tube, and the sample was boiled in a sand bath for about 3 min until the agar had dissolved and orange fumes were no longer visible indicating that the sample had been fully oxidised. The solution was then poured through a filter into a sterile tube and made up to a final volume of 5ml

with HPLC water. The metal concentration was measured by atomic absorption spectrophotometry.

Metal quantification was performed on aqueous samples by AAS using an air-acetylene flame on a Varian SpecrAA-100 spectrophotometer (Varian Instruments, Walton-on-Thames, Surrey, UK) with automatic background correction. Standard solutions of each metal, diluted in 1M nitric acid, were used to calibrate the instrument. The specific absorption due to Cu was determined at 324.7 nm with a 0.5 nm slit width and a 4 mA lamp current. All measurements were performed by Mr M. O'riley at Cardiff University.

2.8 Extraction and purification of nucleic acids

2.8.1 Genomic DNA extraction

A large plate (90mm) of nematodes was washed off with M9 (5ml) and transferred to a 15ml Falcon tube. The tube was centrifuged for 2 min at 2000g and the supernatant was removed. This wash step was repeated with M9 (5ml) and the nematodes were frozen at -20°C. After thawing at room temperature (RT), genomic lysis buffer (450µl) and Proteinase K (0.125mg) was added to digest proteins and nucleases. The sample was incubated at 65°C for 30 min and mixed every 5 min. Another aliquot of proteinase K (0.125mg) was added and the sample was incubated for a further 30 min at 65°C with mixing every 5 min.

The sample was cooled to RT and RNAse A (0.05mg) was added. The mix was incubated at 37°C for 30 min and shaken slowly. Phenol (500µl) was added and mixed by vortex. The sample was then centrifuged at 14,000g for 30 min. Phenol:chloroform:isoamylalcohol (25:24:1, 500µl) was added to the supernatant and mixed gently for 30 min, then centrifuged at 14,000g for 5 min. Chloroform: isoamylalcohol (24:1, 500µl) was added to the supernatant and the sample was mixed gently for 30 min. The sample was then centrifuged for 5 min at 14,000g. The supernatant was removed and 96% ethanol (900µl) was added. The mixture was left O/N at RT, and then centrifuged at 14,000g for 10 min. The supernatant was removed

and the pellet was washed in 70% ethanol (500µl). Finally the pellet was resuspended in 10mM Tris, pH8, (200µl).

2.8.2 Total RNA extraction

Total RNA was extracted from nematodes using the standard Tri-reagent (Sigma) protocol. Nematodes were taken from the -80°C freezer and allowed to thaw on ice for 30 min. An equal volume of glass beads (acid washed) was added which were used to break open the nematodes. Tri-reagent (1ml per 100ul nematodes) was added to extract the RNA. The sample was mixed by vortex for 2.5 min and the supernatant was transferred to a fresh 1.5ml microcentrifuge tube.

After adding Tri-reagent the samples were left to stand at RT for 5 min before adding 200µl of chloroform/ml Tri-reagent to precipitate proteins. After mixing for 15 seconds the sample was allowed to stand at RT for 15 min. The mixture was centrifuged at 12,000g for 15 min (4°C) and the upper aqueous phase was transferred to a fresh microcentrifuge tube. *Iso*-propanol (500µl per ml Tri-reagent) was added to precipitate the RNA and the sample was left to stand at room temperature for 10 min before centrifuging at 12,000g for 10 min (4°C). The supernatant was removed and the pellet was washed with 75% ethanol (1ml). This was then mixed by vortex and centrifuged at 12,000g for 5 min (4°C). The supernatant was removed and the pellet was air-dried for about 5 min, before resupending in HPLC water (100µl). The RNA was stored at -80C until use.

2.8.3 Purification of RNA

RNA was purified using the Qiagen RNeasy kit as follows. The RNA sample (≤100µg) was thawed on ice and adjusted to a volume of 100µl with RNase-free water. Buffer RLT (350µl) was added and after mixing, 100% ethanol (250µl) was added and mixed by pipette. The sample was immediately applied to the RNeasy column in a 2ml collection tube. After centrifugation at 8000g for 15 sec the flow-through was discarded. The RNeasy column was transferred to a new 2ml collection tube and buffer RPE (500µl) was added. The column was spun at 8000g for 15s to wash the column and the flow-through was discarded. The wash step was repeated with another aliquot of Buffer RPE (500µl) and the column was centrifuged at 8000g

for 2 min to dry the RNeasy silica-gel membrane. The RNeasy column was placed in a fresh 1.5ml tube and centrifuged at full speed for 1 min, to ensure complete removal of the buffer. The column was transferred to a final 1.5ml tube and RNase-free water (30µl) was added directly to the RNeasy silica-gel membrane. The sample was centrifuged at 8000g for 1 min to elute the RNA. To increase yield a further aliquot of RNAse-free water (30µl) was added and the column was again centrifuged at 8000g for 1 min.

2.8.4 Purification of mRNA from total RNA

mRNA was isolated from total RNA samples using the Amersham Biosciences mRNA purification kit. For this an oligo-(dT) cellulose spin column was used according to the manufacturer's instructions. In brief, total RNA was added to a cellulose bed (a solid-phase matrix coupled to a dT oligomer) with a high salt buffer. The mRNA hybridised to the column via its poly (A^+) under high salt conditions and was then eluted by lowering the ionic strength and so breaking the dT oligonucleotide and poly (A^+) mRNA association. The whole procedure was repeated, to subject the RNA to another round of purification. Samples were precipitated (with 100µl sample buffer, 10µl glycogen and 2.5ml ethanol/ml sample) at -80°C O/N and then resuspended in 16µl HPLC water.

2.8.5 Phenol-chloroform extraction

To remove protein contaminants from DNA or RNA a phenol-chloroform extraction was used. The purification process was performed by the addition of an equal volume of phenol (pH8.0 for DNA and pH4.0 for RNA) followed by mixing and centrifugation at 13,000g for 3 min. The upper layer was carefully removed and transferred to a fresh 1.5ml tube. This wash step was repeated with another equal volume of phenol and then twice with chloroform. The upper aqueous layer, containing the purified DNA or RNA, was removed and transferred to a final 1.5ml tube and the DNA or RNA was ethanol precipitated O/N (***Section 2.8.6***).

2.8.6 Ethanol precipitation

In order to concentrate DNA and RNA samples, an ethanol precipitation step was performed. The DNA or RNA was precipitated O/N at -80°C by addition of 2 volumes of 100% ethanol and 0.1 volumes of 3M Sodium Acetate (pH4.8). The DNA or RNA was recovered by centrifugation at 13,000g for 15 min (4°C). The supernatant was removed and the pellet was air-dried for 2-5 min before resuspending in an appropriate volume of HPLC H_2O.

2.9 Visualisation and quantification of nucleic acids

2.9.1 Agarose gel electrophoresis

DNA was routinely analysed by agarose gel electrophoresis (agarose 1.0-2.5% (w/v) in TAE buffer containing 0.5μg/ml ethidium bromide (EtBr)). Samples were loaded into the wells of the gel after mixing with 20% (v/v) DNA gel loading dye. Electrophoresis was carried out in a Pharmacia GNA-100 tank, with a Pharmacia EPS-200 power pack at a constant voltage of 80-120V for 20-60 min in 1X TAE buffer. Appropriate DNA molecular weight markers (***Table 2.6***) were used to determine the size of DNA fragments visualised with a UV light source. A UV trans-illuminator camera was used to photograph the gels. For RNA gel electrophoresis gel tanks, boats and combs were soaked in 1% SDS overnight and 1X TAE was double autoclaved to minimise RNAse activity.

2.9.2 Extraction of DNA from agarose gels

After running 50μl of the DNA sample on an agarose gel, the DNA bands, visualised under UV light, were carefully excised using a sterile scalpel. A 1ml filter tip was cut using a sterile scalpel just below the filter and placed into a 1.5ml microcentrifuge tube. The excised DNA bands were placed into the cut filter tip and centrifuged at 20,000g for 7 min at RT. The DNA was then extracted by phenol-chloroform (as described in ***Section 2.8.5***, except that all centrifugation steps were performed at 14,000g for 4 min at RT) and ethanol precipitated (***Section 2.8.6***).

Alternatively DNA was extracted from agarose gels using the Qiagen QIAquick Gel extraction kit. The DNA fragment was excised using a sterile scalpel and 3 volumes of Buffer QG was added. After the agarose gel was dissolved (by incubating at 50°C for 10 min) 1 volume of *iso*-propanol was added to precipitate RNA. The mix was placed in a QIAquick spin column and bound by centrifugation at 13,000g for 1 min. The column was washed with Buffer PE (750µl) and eluted with Buffer EB (50µl) by centrifugation.

2.9.3 Quantification of nucleic acids using a spectrophotometer

The absorbance of nucleic acids at a wavelength of 260nm was measured using the Vitrospec 2100 pro UV spectrophotometer or the Genequant (Pharmacia). An absorbance at 260nm (A_{260}) of 1, in a 1cm standard cuvette, is equivalent to 50µg/ml of dsDNA or 40µg/ml ssDNA and RNA (Sambrook *et al.*, 1989). By measuring the absorbance at 230nm and 280nm the purity of the sample could be determined. The A_{260}/A_{280} ratio of pure DNA or RNA should be between 1.8 and 2.0, respectively. Proteins, phenols and most carbohydrate contaminants increase the A_{280} and so reduce the ratio A_{260}/A_{280} (Sambrook *et al.*, 1989). Likewise, a ratio of A_{260}/A_{230} less than 2.0 is considered to be indicative of ethanol or salt contamination.

2.9.4 Ethidium bromide spot assay

EtBr intercalates DNA and RNA and can be used to quantify small amounts of nucleic acids when unincorptated nucleotides have been removed. The sensitivity range of this technique is between 1-5ng of nucleic acid. Standard dilutions were made of a known marker (λH3 diluted from 100ng/µl to 0.2ng/µl). The unknown sample was diluted four times (1 in 5 to 1 in 40). On para film 2ng/µl EtBr (0.5µl) was mixed with each diluted standard and sample (0.5µl). The UV transluminator was used to visualise and estimate the quantity of DNA present.

2.10 Polymerase chain reaction (PCR)

2.10.1 Production of Taq polymerase

The enzyme *Taq* (*Thermus aquaticus)* polymerase was used to amplify numerous copies of target DNA by PCR. *Taq* polymerase was the first thermostable enzyme to be used in PCR (Saiki *et al.,* 1988) and still remains an efficient way of carrying out successful PCR. To produce *Taq* polymerase, DH5α cells containing the pTaq vector were grown O/N at 37°C in LB+Amp broth (6ml). The culture was centrifuged at 2000g for 10 min and the supernatant was removed. The pellet was resuspended in fresh LB+Amp broth (6ml). Of this 0.25ml was used to inoculate fresh LB+Amp broth (500ml) and the culture was grown to an optical density at 600nm (OD_{600}) of 0.2. 1M IPTG (500µl) was added and the culture was allowed to continue growing for 12-16 h at 37°C.

Strips of dialysis tubing were boiled in dH_2O for 10 min and stored in a falcon tube containing HPLC water. Cells were harvested by centrifugation at 600g for 10 min (4°C) and were kept on ice from thereon. The supernatant was removed and the pellet was mixed by vortex before resuspending in fresh LB+Amp broth (100ml). The centrifugation step was repeated and the pellet was resuspended in 100ml Buffer A (***Table 2.2***). The mixture was then centrifuged at 600g for 10 min (10°C) and the supernatant was removed. The pellet was resuspended in pre-lysis buffer (50ml) and incubated at room temperature for 15 min. Lysis buffer (15ml) was added and mixed by swirling then incubated at 75°C for 1 hr before centrifugation at 10,000g for 20 min (4°C).

The supernatant was transferred to a new container and 50% (w/v) Ammonium sulfate was slowly added whilst stirring. The solution was centrifuged at 10,000g for 20 min (4°C) and the supernatant was removed. The pellet was resuspended in Buffer A (20ml) and placed in the dialysis tubing, securing each end with two sealer clips. The tubing was placed in a beaker containing 1L of storage buffer. The protein mix was dialysed against two changes of storage buffer (allowing about 12 h between each buffer change). The protein was aliquoted and stored at -20°C (or -80°C for long-term storage). Viability of the *Taq* polymerase activity was tested at different dilutions against commercial *Taq* (Promega).

2.10.2 Single worm PCR (SWPCR)

Individual *C. elegans* were placed into a PCR tube containing 2.5µl of the worm lysis buffer (***Table 2.2***). The sample was mixed and then pulsed down by centrifugation. The tube was incubated at -80°C for at least 30 min, 65°C for 1 hour and then 95°C for 15 min. The 2.5µl sample was used as a template in a standard PCR (***Section 2.10.5***).

2.10.3 Reverse transcription –PCR (RT-PCR)

The RT-PCR method consisted of two distinct steps, the first being cDNA synthesis from isolated total RNA, using reverse transcriptase (see below), followed by amplification of the desired cDNA target by PCR using specific primers (***Section 2.10.6***).

The following mix was made up on ice: 10mM dNTPs (1µl), 100pmol/µl Oligo dT (1µl), 100pmol/µl random hexamers (1µl), total RNA (2µg) and dH_2O to a final volume of 10µl total. This was heated at 70°C for 10 min, and then returned to ice. 5X Reaction buffer (4µl), 0.1M DTT (2µl), RNasin (0,5µl) and HPLC water (2.5µl) was added. The sample was then mixed and M-MLV Reverse transcriptase (1µl) was added. The mixture was incubated at room temperature for 10 min then 37°C for 50 min and finally heat inactivated at 80°C for 10 min.

2.10.4 Primer design for standard PCR

Specific primers to a target sequence were designed with two main considerations, specificity and annealing temperatures, both of which are dependent on sequence content and length. A 16bp primer would occur less than one in a billion base pairs of genomic template (Sambrook et al., 1989) while primers larger than 25bp have reduced specificity (particularly at the 5′ end), thus primers of 19bp were generally designed. The sequence would optimally have 50-60% GC content, with a pair of primers having a closely matched GC content. The annealing temperature of a primer is related to its melting temperature (T_m) and is estimated by the following equation:

$$T_m = 4 \text{ x } (GC) + 2 \text{ x } (AT)$$

This T_m value is used as a guide for optimising conditions in subsequent polymerase chain reactions (PCR) for each pair of primers (see ***Appendix A5*** for a list of primers used and their optimal conditions). In order to minimise mispriming, primers were designed using software such as Primer 3 (Whitehead Institute for Biomedical Research). This was used to check the risk of hairpin-loop formation (self-complimentary sequence) and primer-dimer formation (primer-primer complimentary sequences).

2.10.5 PCR amplification

The following standard mix was made up for PCR reactions: dNTPs (0.2mM), Forward primer (1pmoles), Reverse primer (1pmoles), 10% (v/v) of 10X *Taq* buffer, *Taq* polymerase (0.125U/µl), DNA/RNA template (1µg) or purified plasmid (0.1-1ng) and HPLC H_2O (filter sterilized) to an appropriate final volume (20µl or 50µl). The final concentration of $MgCl_2$ was adjusted in the range of 1-4mM, according to the template and primer set used. Unless stated otherwise, all reactions were in a total volume of 20µl and contained 2.5mM $MgCl_2$. PCR amplifications were performed in a Techne Flexigene thermocycler as below, with the cycling conditions being optimised for each set of primers and template used.

- 95°C for 5 min
- 95°C for 1 min
- X°C for 1 min
- 72°C for Z min
- 72°C for 10 min

Y Cycles

- Hold at 4°C.

X= the annealing temperature of the specific primers to the template (see ***Table 7.1***)

Z= the extension time where 1 minute allows amplification of about 1Kb of DNA

Y= the number of PCR amplification cycles (20-35)

2.10.6 Purification of PCR products

On occasions it was necessary to purify PCR products prior to downstream applications such as for ligations, sequencing or sequential digests with incompatible buffers in a double restriction digest.

The DNA was purified using the QIAquick PCR Purification kit (Qiagen). The DNA and 5 volumes of buffer QG were to a QIAquick spin column. The DNA was bound to the column matrix by centrifugation at 13,000g for 30-60 seconds. The column was washed by addition of Buffer PE (750µl). The flow though was discarded and the sample was centrifuged for an additional minute. The DNA was eluted in a clean microcentrifuge tube by adding Buffer EB (50µl) and centrifuging at 13,000 rpm for 1 min.

2.11 Quantitative PCR (QPCR)

2.11.1 Introduction to QPCR

QPCR amplifications were performed to assess the expression levels of specific genes, using TaqMan® probe technology on the ABI Prism® 7700 Sequence Detection System. This method is based on PCR where accumulation of PCR products is measured by monitoring fluorescent levels through a dual-labelled fluorogenic probe (TaqMan® probe), allowing the quantification and expression profiles of genes to be determined.

The probe is dual-labelled, with the reporter dye 6-carboxyfluorescein (FAM) at the 5′ termini, and the universal quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ termini. The close spatial proximity of the universal quencher to the reporter dye disrupts the observable fluorescent emission from the reporter dye. It does this by the use of Fluorescence Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton.

Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation and the reaction cools, the primers anneal to the DNA. During amplification the hybridised probe is digested by the 5′ → 3′-exonuclase activity of the *Taq* DNA polymerase in the primer extension phase of amplification. This separates the quencher from the reporter, allowing the reporter to emit its light energy

in an excited state (Nasarabadi et al., 1999) which can now be observed as an increase in fluorescence which is monitored by computer (***Figure 2.1***). The relative level of gene expression can be quantified because the more times the denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected.

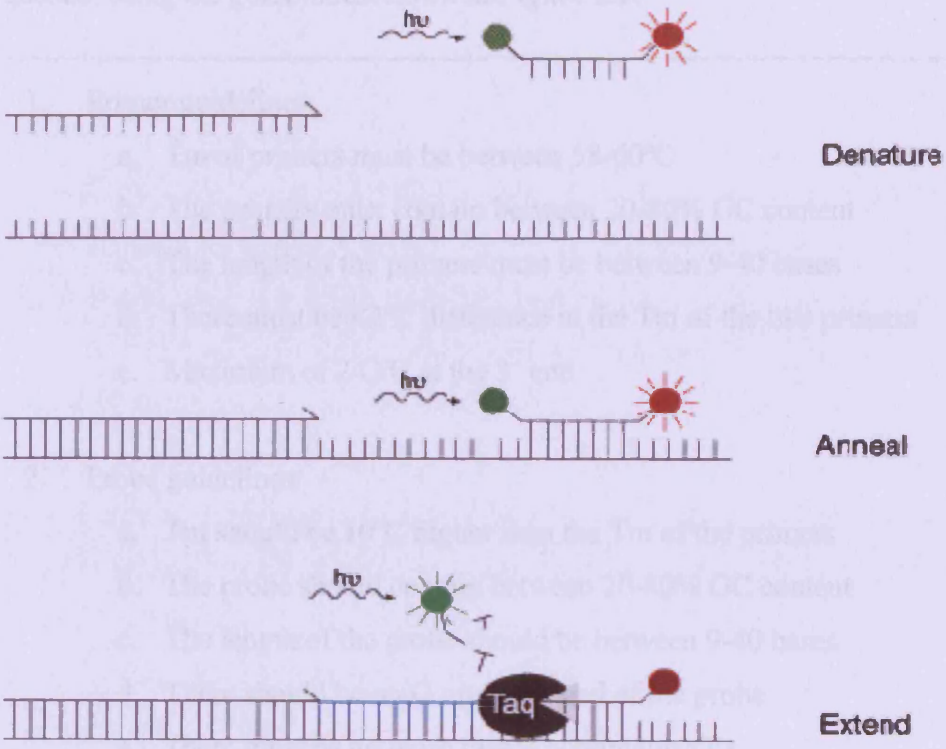

Figure 2.1 *Schematic view of the TaqMan® probe technology. Before the probe anneals, energy is transferred from the reporter, a short-wavelength fluorophore (green) to the quencher, a long-wavelength fluorophore (red). When the probe and primer anneal,* Taq *polymerase adds nucleotides to the template strand, causing the reporter fluorophore to be released, making it detectable (adapted from www.probes.com).*

2.11.2 Primer and probe design

Primers and TaqMan® probes for use in the TaqMan® quantification system were designed using the Primer Express™ software package (PE Applied Biosystems). The primers were synthesised by MWG Biotech whilst the TaqMan® probes were synthesised by Eurogentec with the reporter dye FAM covalently linked to the 5′ ends and the quencher dye TAMRA at the 3′ ends. Primers were designed to amplify an amplicon of 50 to 150bp in length, with the TaqMan® probe hybridising to the sequence; using the guide-lines shown in ***Figure 2.2***.

1. Primer guidelines:
    a. Tm of primers must be between 58-60°C
    b. The primers must contain between 20-80% GC content
    c. The length of the primers must be between 9-40 bases
    d. There must be <2°C difference in the Tm of the two primers
    e. Maximum of 2 G/C at the 3′ end

2. Probe guidelines:
    a. Tm should be 10°C higher then the Tm of the primers
    b. The probe should contain between 20-80% GC content
    c. The length of the probe should be between 9-40 bases
    d. There should be no G on the 5′ end of the probe
    e. There must be no more than 4 contiguous G's
    f. The probe must not contain more G's than C's
    g. The probes were designed over intron/exon boundaries to minimise genomic contamination

3. Amplicon:
    a. The amplicon should be between 50-150bp in length
    b. The 3′ end of the primers should be as close to the probe as possible without overlapping

Figure 2.2 *Guidelines for QPCR primers and TaqMan probe design*

2.11.3 Preparation of standards

Stock plasmids that contained an insert corresponding to the target sequence to be amplified for quantification purposes, were prepared (***Section 2.13.1***), purified and concentrated by ethanol precipitation (***Section 2.8.6***). Quantification and purification assessments were performed by spectrophotometry (***Section 2.9.3***) and agarose gel electrophoresis (***Section 2.9.1***). A standard dilution series of calibration standards were prepared by diluting each purified plasmid to a known concentration, in the range of 1ng/µl to 100ag/µl.

2.11.4 Preparation of samples

Nematodes were washed off agar plates using M9 into 15ml falcon tube. The tube was centrifuged for 2 min at 2000g and the supernatant was removed. This wash step was repeated with M9 (5ml) and the supernatant was reduced to a minimal volume. In a 2L flask the nematodes were cultured in S-basal media (750ml) inoculated with OP50 (20ml) and LB broth (250ml). The flask was incubated at 20°C with shaking at 200rpm. The nematodes were titred daily until the population had reached sufficient numbers (approximately 50,000 mixed stage for each extraction). After centrifugation at 2000g for 2 min, the supernatant was removed to a minimal volume and the culture was split into 15ml falcon tubes. The nematodes were then exposed to 0, 100, 500 and 2000µM $CuSO_4$ for 24 hours with 5 biological replicates at each concentration.

Total RNA was extracted from non-exposed and exposed *C. elegans* (***Section 2.8.2***). The RNA was purified and concentrated by ethanol precipitation (***Section 2.8.6***) and 2µg was used to generate cDNA in a RT reaction using specific primers to the target sequence (***Section 2.10.4***). Each cDNA sample was diluted 1:10 with HPLC H_2O prior to quantitative analysis.

2.11.5 Optimisation of QPCR

Optimisation of both primer and probe concentrations was required for TaqMan® probe PCR quantification. The amplification reaction consisted of the following: purified plasmid DNA template (250pg), dNTPs (1mM), 10% (v/v) of 10X *Taq* buffer (containing light-sensitive ROX), self made *Taq* polymerase (1.6µl/ml), forward primer (900nM), reverse primer (900nM), probe (200nM) and HPLC H_2O (filter sterilized) to a final volume of 25µl.

The PCR reactions were performed in 96-well PCR plates, using optical lids, and subjected to the following cycling parameters using the ABI Prism® 7700 sequence detection system:

- 50°C for 2 min
- 95°C for 10 min
- 95°C for 15 sec
- 60°C for 1 min

} 40 Cycles

Product formation was monitored at the end of each extension step by measuring the fluorescence emitted from the TaqMan® probes and Ct values were calculated.

2.11.6 TaqMan® QPCR amplifications

The amplification reactions were performed using the optimised gene specific conditions. Calibrations standards and samples were quantified in parallel. A series of 8 standard dilutions of plasmid DNA (ranging from 1ng/µl to 100ag/µl) were prepared (***Section 2.12.3***) and analysed in triplicates.

A standard curve was obtained and used to generate a regression line, by plotting the cycle number required to attain a threshold fluorescence. Five biological replicates were prepared for each test sample, and for each biological replicate the cDNA samples were analysed in triplicates. Diluted cDNA samples (prepared in ***Section 2.12.4***) were used in the quantitative PCR process (2.5µl of diluted (1:10) cDNA per 25µl quantitative PCR reaction volume). Using the standard curve obtained, a regression line was generated, extrapolating each sample over the standard range.

2.12 DNA modifications

2.12.1 Restriction digests of DNA

DNA samples were digested with commercially available restriction enzymes. The total volume of a restriction digest was made up to 20µl with HPLC H_2O and consisted of 200-500ng purified plasmid DNA, 5U of each restriction enzyme and 10% (v/v) 10X digestion buffer (chosen for optimised efficiency for both restriction enzymes in a double digest). The components were gently mixed and briefly centrifuged to bring the contents to the bottom of the tube before incubating at 37°C for 2 hours. The reaction was heat inactivated at 65°C or 80°C (depending on the enzyme) for 20 min before visualising the result by electrophoresis on an agarose gel (***Section 2.9.1***).

2.12.2 Blunt-ending DNA products

The 5′ to 3′ polymerase activity of *Pfu* DNA Polymerase was used to fill in 5′ overhangs generated by restriction digests. The following mix was made: 1 to 5µg digested vector, 1M Tris-HCl, pH 7.5 (1.5µl), 0.1M $MgCl_2$ (2.5µl), 0.1M β-Mercaptoethanol (2.5µl), 1mM dNTPs (5µl) and *Pfu* DNA polymerase (2.5U). This was made up to a total volume of 25µl with HPLC water and incubated at 12°C for 1 h then inactivated at 68°C for 10 min. The sample was purified by phenol-chloroform extraction (***Section 2.8.5***) and ethanol precipitation (***Section 2.8.6***).

2.12.3 Dephosphorylation of terminal DNA ends

DNA (10 to 20µg) was digested (***Section 2.12.1***) with the appropriate enzyme and the sample was purified by ethanol precipitation (***Section 2.8.6***). To the linearised DNA, 10X CIAP buffer (50µl) was added with CIAP (0.01U for 5′ recessed or blunt ends) using the formula that 2µg of linearised plasmid DNA, 5Kb in length, contains about 1.4pmoles of 5′-terminal phosphate residues. The mix was incubated at 37°C for 15 min and 56°C for 15 min. A second aliquot of CIAP (0.01U) was added and the incubation step was repeated. CIAP stop buffer (300µl) was added and the sample was then purified by phenol-chloroform extraction (***Section 2.8.5***) and ethanol precipitation as described in ***Section 2.8.6*** except that 0.5 volumes of 7.5M

Ammonium Acetate (pH5.5) and 2 volumes 100% ice-cold ethanol were added to the final phase.

2.13 Cloning and expression of target genes

2.13.1 Purification of plasmid DNA

For the purification of plasmid DNA, a Wizard Plus SV Miniprep DNA Purification System (Promega) was used according to the supplier's instructions. LB (5-10ml) with an antibiotic component was inoculated with a single colony that contained the desired plasmid DNA and grown O/N at 37°C shaking at 200rpm.

The culture (5ml) was centrifuged, and cell resuspension solution was added to the pellet. EDTA in the cell resuspension solution makes the bacterial outer membrane permeable. It also contains Mg^{2+} which chelates DNAses, preventing their action and the RNAse A degrades any RNA present in the sample. The cells were then lysed by adding a solution containing 0.1M NaOH, which denatures proteins and disrupts chromosomes as well as cleaving RNA. It also contains 1% SDS, a detergent which lyses the cell membrane, allowing plasmids to escape. Alkaline protease solution was added prior to incubation, which breaks down bacterial proteases. Neutralisation solution was then added which contains guanidine hydrochloride and potassium acetate which form a precipitate with the proteins and chromosomal DNA while the plasmids remain in solution. Glacial acetic acid in this solution neutralises the alkali from the cell lysis step. The reaction was mixed and centrifuged, resulting in a clear supernatant containing the plasmid. The precipitate formed contains proteins, chromosomal DNA and cell debris.

The clear supernatant was transferred into a Wizard® *plus* SV miniprep spin column. Where under high salt conditions, the nucleic acids bind to the resin in the spin column. Column wash was added and centrifuged to remove proteins and salts. The wash solution contains ethanol and a small amount of salt, so that mono-nucleotides are removed but plasmid DNA remains bound. DNA was released from the resin by the addition of nuclease free water (pre-heated to 65°C) and was collected by centrifugation.

2.13.2 Large scale plasmid DNA purification

For large scale purification of plasmid DNA the Wizard Plus SV Maxiprep DNA Purification System (Promega) was used. LB media (about 10ml) with an antibiotic component was inoculated with a single colony containing the desired plasmid DNA and grown O/N at 37°C in an orbital shaker (200rpm). From the O/N culture 100µl was transferred to a flask containing 500ml fresh LB with antibiotic and incubated O/N with shaking at 37°C. The culture was centrifuged, resuspended, lysed, neutralised and centrifuged again. The supernatant was placed onto a column and washed then the plasmid DNA was eluted by gravity flow. To the eluant, *iso*-propanol was added to precipitate the plasmid DNA and the mixture was centrifuged to form a pellet. The pellet was washed with 70% ethanol before resuspending in RNase free water (provided in the kit).

2.13.3 Ligation of DNA fragments into vectors

PCR products excised from agarose gels according to ***Section 2.9.2*** were ligated into vectors using a 2X ligation kit (Promega). The ligation mixture contained 50% (v/v) 2X ligation buffer, 3U T4 ligase and 50ng vector made up to 10µl with nuclease-free water. An appropriate amount of purified DNA fragment was added according to the equation below:

$$\frac{\text{50ng vector x size (Kb) of insert}}{\text{Size (Kb) of vector}} \times \text{insert :vector molar ratio (usually 3:1)} = \text{ng of insert}$$

The ligation was incubated at 4°C O/N or at 12°C for 2 days.

2.13.4 Preparation of competent cells

Competent cells were prepared using the standard $CaCl_2$ protocol (Sambrook *et al.*, 1989). LB broth (10ml) with an appropriate antibiotic was inoculated with the bacteria and left shaking at 37°C O/N. The O/N culture was used to inoculate 1L LB broth (with antibiotic) and shaken at 30°C for 7 h. Approximately 20ml of the culture was used to inoculate 500ml of fresh LB broth (plus antibiotic), which was then grown for about 3 h to an OD_{600} of 0.35-0.40 (where cells enter the early/mid log phase). The cells were placed at 4°C for all subsequent steps and centrifuged at 300g for 10 min at 4°C. The supernatant was discarded and the pellet was carefully resuspended in chilled, sterile 50mM $CaCl_2$ (500ml) and left on ice for 30 min. The cells were then re-centrifuged as before and the supernatant discarded. The pellet was resuspended in chilled, sterile 50mM $CaCl_2$ (100ml) and transferred into 100µl aliquots. After snap freezing in liquid N_2 the cells were stored at –80°C.

2.13.5 Transformation into competent cells

Competent cells were allowed to thaw on ice for 5 min before placing 100µl into a pre-chilled Falcon tube and adding 10µl ligation mixture (***Section 2.14.3***). After mixing gently, the Falcon tube was placed on ice for 20 min. The sample was then heat shocked in a 42°C water bath for 45 seconds. The transformation mixture was immediately placed on ice for 2 min and 900µl SOC (pre-warmed to 42°C) was added. The tube was mixed by flicking and incubated for 1-1.5 hours at 37°C with shaking (200rpm). After the incubation, 100µl transformed cells were spread over an agar plate with an appropriate antibiotic and left to grow for 16-20 h at 37°C.

2.13.6 Selection of positive clones

Three methods were commonly used to identify the most likely positive clones to contain the inserted DNA of interest. To identify colonies of transformed cells containing constructs, a simple blue/white screening process was employed, involving the use of blue/white indicator plates. A successfully ligated insert in the pBlueScript or pGEM-T vector interrupts the coding sequence for the β-galactosidase. Therefore, transformants containing inserted DNA mostly produce white colonies (positive clones), whilst empty vectors yield blue colonies (negative clones).

Blue/white indicator plates were prepared by adding IPTG (10mM) and X-gal (20mg/ml) to LB+Amp agar. The transformation was spread on the plates and incubated at 37°C O/N. Positive clones identified by blue/white screening were further validated to contain constructs of the correct size by a more specific selection process as described below.

The second method was to directly PCR each colony using vector primers (or specific primers where possible) and to measure the product size by agarose gel electrophoresis (***Section 2.9.1***). Individual colonies to be investigated were transferred with a sterile pipette tip directly into a PCR mixture (***Section 2.10.6***). The same pipette tip used to pick the colony and place in the PCR reaction mixture was used to make a stock by streaking an agar plate which was then incubated O/N at 37°C.

A third method relied on extracting the plasmid DNA (***Section 2.13.1***) and performing a PCR (***Section 2.10.5***) or double restriction digest (***Section 2.12.1***) on the plasmid DNA to confirm the size of insert present.

2.14 Nucleic acid sequencing

2.14.1 Big Dye sequencing reaction

ABI Big Dye V3.1 was pre-diluted as follows: Big Dye (40µl), 5X Buffer (40µl) and HPLC water (160µl). The following components were mixed in a sterile 0.2ml PCR tube: diluted Big Dye (6 µl), appropriate Primer (15pmol), plasmid DNA (290ng) or PCR product (60ng) and HPLC water (to final volume of 15µl). The following amplification was performed using a Techne Flexigene thermocycler.

- 96°C for 3 min
- 96°C for 15 sec
- 50°C for 10 sec
- 60°C for 3 min
- 72°C for 3 min

35 Cycles

- 4°C Hold

2.14.2 Iso-*propanol sequencing clean-up*

Labelled sequencing products were precipitated using iso-*propanol.* The 15µl PCR reaction was transferred to a 0.5ml PCR tube (thick-walled). 90% *iso*-propanol (90µl) was added and the mix was incubated for 15 min at RT in the dark. The mixture was centrifuged at 14,000g for 30 min and the supernatant was carefully removed. 70% *iso*-propanol (150µl) was added and the sample was centrifuged at 14,000g for 15 min. The supernatant was carefully removed and the pellet air-dried in the dark under vacuum for 20 min. The products were sequenced by the technical support unit (Molecular Biology Support, Cardiff School of Biosciences) on a capillary ABI-3100 automated DNA sequencer (model 4000L) and analysed with the Chromas package, version 2.21.

2.14.3 Sequence identification and analysis

Following the sequencing of the DNA, the nucleotides and amino acid sequences were subject to bioinformatic analysis. DNA sequences were compared to all database entries, searching for nucleotide similarities (BlastN searches), using the Blast search analysis programme and for amino acid similarities in all translated reading frames (BlastX searches), incorporated into the EBI on-line software suite (http://www.ebi.ac.uk).

The computer programme DNAsis (Hitachi) and the BioEdit sequence alignment editor (version 4.8.10) were used to analyse a DNA sequence, including procedures such as open reading frame analysis, translation, and multiple alignment generation and subsequent consensus sequence generation.

Chapter 3

Investigations into copper toxicity in *C. elegans*

Chapter 3

Investigations into copper toxicity in *C. elegans*

3.1 Introduction

The model organism *Caenorhabditis elegans* (*C. elegans*) was utilised in order to investigate the whole organism response to copper (Cu) toxicity. Initially atomic absorption spectrophotometry (AAS) was performed to measure the nitric acid extractable concentration of Cu in agar plates, with and without Cu in the bacterial food source. Cu EC50 and LC50 experiments were carried out to determine the range of sub-lethal and lethal concentrations of Cu in *C. elegans* to facilitate further studies. EC50 is the concentration at which reproduction is reduced to 50% and LC50 is the concentration at which 50% of the animals die. The whole organism response and phenotypic effects of Cu on *C. elegans* was characterised.

3.2 Atomic Absorption spectrophotometry (AAS)

C. elegans were maintained on NGM agar (***Table 2.2***) in Petri dishes with the *Escherichia coli* (*E. coli*) strain OP50 as a food source. NGM agar contains basal amounts of Cu and as additional Cu was added in the form of $CuSO_4.7H_2O$, it was important to determine the concentration of actual bio-available Cu in the agar. This involved measuring the nitric acid extractable concentration of Cu in the agar by AAS (***Section 2.7***).

It was necessary to evaluate whether adding $CuSO_4$ to the bacterial food source, OP50, significantly altered the Cu concentration. The Cu concentration was compared in plates with and without streaking the OP50 culture containing $CuSO_4$ onto the NGM agar (***Section 2.7***). The nitric acid extractable concentration of Cu was measured in agar plates over a range of nominal $CuSO_4$ concentrations. At each concentration five replicates were measured and the average results are shown in ***Figure 3.1.***

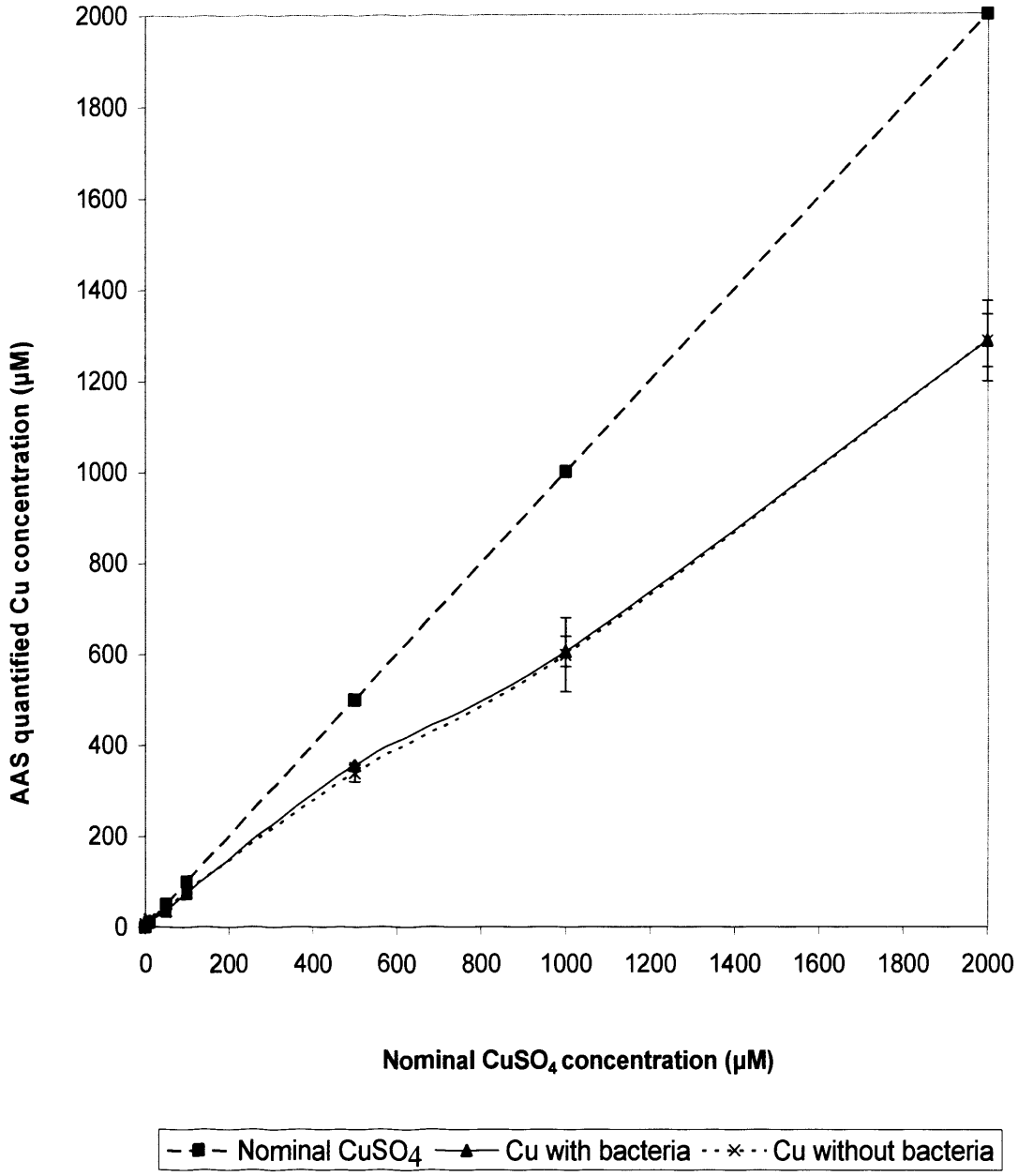

Figure 3.1 *AAS results showing the nitric acid extractable concentration of Cu against the nominal amount of $CuSO_4 .7H_2O$ added to the NGM agar with and without the bacterium OP50 containing $CuSO_4$. The nominal concentration of $CuSO_4$ is also shown for comparison, ± SEM (n=5).*

The AAS results show that there is no observable difference in the nitric acid extractable concentration of Cu in the agar with and without streaking OP50 containing $CuSO_4$ onto the agar plates. The nitric acid extractable concentration of Cu in the agar plates is up to 40% less than the nominal concentration of $CuSO_4$ added to the agar plates.

For the purpose of these studies, the stated amount of Cu used in subsequent experiments will be expressed in terms of the $CuSO_4.7H_2O$ concentration added rather then the nitric acid extractable concentration of Cu, unless otherwise stated. For a table of extractable Cu concentrations calculated from the nominal amounts of $CuSO_4.7H_2O$ added see Appendix ***Table A.1***.

3.3 Copper and iron toxicity in *C. elegans*

3.3.1 *Introduction to copper and iron homeostasis*

In eukaryotic organisms, Cu and iron (Fe) homeostasis are intertwined (Freitas *et al.*, 2003), thus the concentration of Fe in the NGM agar was also measured. There is a well recognised link between Cu and Fe metabolism which is illustrated by the Cu dependence of high affinity Fe uptake in yeast and mammals, with a Cu deficiency resulting in impaired Fe uptake (Herbik, *et al.*, 2002). This dependence is due to the role that Cu plays as a cofactor for the ferroxidase Fet3p (***Figure 3.2***). The *C. elegans* genome contains a Fet3p which is homologous to the iron transport multicopper oxidase FET3 precursor. Studies of Fet3p suggest that this ferroxidase might also play a role in cellular defence against Cu toxicity. One explanation for this observation is that Fet3p may function in Cu detoxification by binding free Cu, thus suppressing the pro-oxidant activity that this metal ion exhibits in the generation of oxygen free radicals (Shi, *et al.*, 2003). Due to this, Fe studies were carried out to determine whether changes in the Fe concentration affected overall Cu homeostasis.

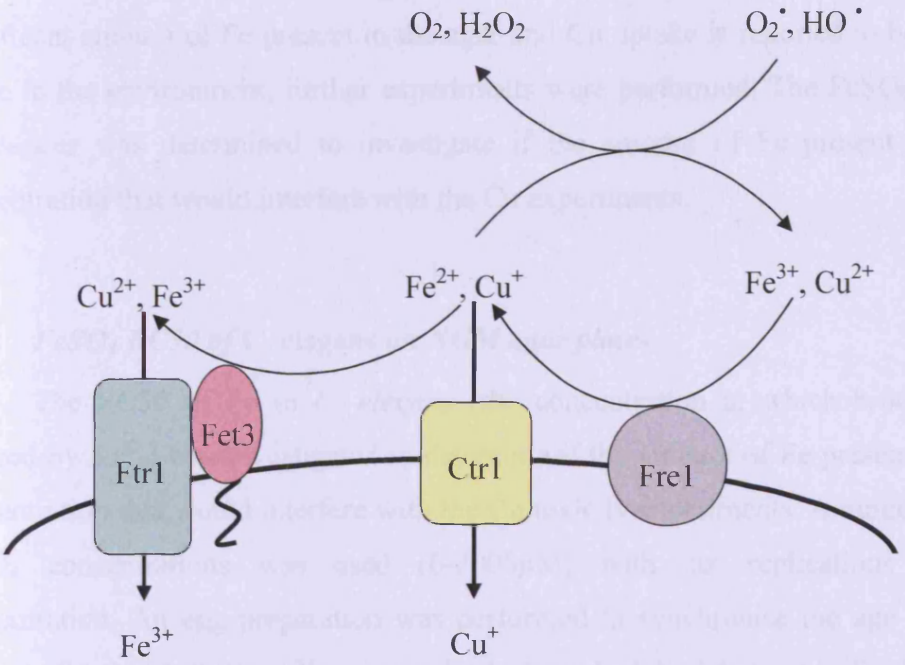

Figure 3.2 *Model of Cu and Fe metabolism in* S. cerevisiae. *The dual roles of Fre1 (metalloreductase) and Fet3 (ferroxidase) are shown. Fre1 generates Fe^{2+} and Cu^{+} for high affinity uptake of both metal ions; Fet3 targets the Fe^{2+} for Fe uptake through ferroxidation and buffers the Cu^{+}/Cu^{2+} ratio through cuprous oxidation. Ctr1and Ftr1 allow import of Cu^{+} and Fe^{3-} respectively into the cell (adapted from Shi* et al. *2003).*

The average amount of Fe present in NGM agar was determined by AAS and is shown below in ***Table 3.1***.

NGM agar	AAS of Fe with bacteria		AAS of Fe without bacteria	
	Fe (µM)	**± *SEM***	**Fe (µM)**	**± *SEM***
Average Fe in $CuSO_4$ plates	70.7	*12.4*	71.6	*12.9*

Table 3.1. *AAS results, showing the nitric acid extractable concentration of Fe in NGM agar plates with and without the bacteria OP50. Results as averages, (n=5) ±.*

The average amount of Fe present in the NGM agar was 71μM. As there is a significant amount of Fe present in the agar and Cu uptake is reported to be affected by Fe in the environment, further experiments were performed. The $FeSO_4$ EC50 in *C. elegans* was determined to investigate if the amount of Fe present was at a concentration that would interfere with the Cu experiments.

3.3.2 *$FeSO_4$ EC50 of* C. elegans *on NGM agar plates*

The EC50 of Fe in *C. elegans* (the concentration at which brood size is reduced by 50%) was investigated to determine if the amount of Fe present was at a concentration that would interfere with the Cu toxicity experiments. A range finder of $FeSO_4$ concentrations was used (0-2000μM) with six replications at each concentration. An egg preparation was performed to synchronise the age of the *C. elegans* (***Section 2.3***). Once the nematodes had reached the L4 stage, individuals were placed onto the test plates. Every 24 hours the nematode was transferred to a fresh NGM agar plate and the total number of progeny produced over a range of $FeSO_4$ concentrations was measured over a 3 day period (***Table 3.2***).

$FeSO_4$ concentration (μM)	**Total Brood Size *(± SEM)***	**Total number of progeny as % of control**
0	191 ± *8.7*	100
20	185 ± *5.2*	97
100	180 ± *3.5*	94
500	171 ± *5.2*	90
1000	116 ± *3.9*	61
2000	96 ± *4.9*	50

Table 3.2 *Amount of progeny produced over a range of $FeSO_4$ concentrations. Results shown as total brood size and as a percentage of control, ± SEM (n=6).*

The $FeSO_4$ EC50 in *C. elegans* over a 3 day period was determined to be 2000μM. As the basal concentration of Fe in the NGM agar was 71μM and the EC50 of *C. elegans* was 2000μM, the level of Fe present in the agar was not as a sufficient concentration to effect subsequent Cu toxicity experiments.

3.3.3 *$CuSO_4$ EC50 of* C. elegans *on NGM agar plates*

The whole organism response to Cu toxicity in *C. elegans* was measured by calculating the EC50. A range finder of $CuSO_4$ concentrations was used (0-1000μM) with six replications at each concentration. An egg preparation was performed to synchronise the age of the *C. elegans* (***Section 2.3***). Once the nematodes had reached the L4 stage, individuals were placed onto the test plates. Every 24 hours the nematode was transferred to a fresh NGM agar plate and the number of progeny was counted every 24 hours for 3 days (***Section 2.4***). The results are shown in ***Table 3.3.***

$CuSO_4$ concentration (μM)	24 hours *(± SEM)*	48 hours *(± SEM)*	72 hours *(± SEM)*	Total Brood Size *(± SEM)*	Total number of progeny as % of control
0	59 ± *5.8*	94 ± *8.2*	94 ± *5.4*	247 ± *11.8*	**100**
1	36 ± *6.8*	82 ± *7.5*	89 ± *1.0*	207 ± *14.9*	**84**
5	33 ± *6.5*	85 ± *6.1*	106 ± *4.3*	224 ± *11.5*	**91**
10	41 ± *5.8*	67 ± *11.3*	107 ± *5.0*	215 ± *19.4*	**87**
25	43 ± *13.4*	77 ± *8.0*	98 ± *0.5*	218 ± *11.5*	**88**
50	41 ± *7.1*	83 ± *6.5*	78 ± *2.0*	202 ± *11.3*	**82**
75	39 ± *3.9*	70 ± *10.1*	57 ± *4.7*	166 ± *14.2*	**67**
100	41 ± *8.9*	48 ± *13.2*	37 ± *3.5*	126 ± *30.3*	**51**
125	31 ± *6.3*	51 ± *6.6*	35 ± *3.0*	117 ± *12.3*	**47**
150	41 ± *5.9*	51 ± *3.2*	18 ± *2.7*	113 ± *9.3*	**46**
200	44 ± *4.4*	18 ± *1.6*	10 ± *0.0*	69 ± *6.2*	**28**
400	48 ± *5.3*	16 ± *4.1*	3 ± *0.0*	67 ± *8.5*	**27**
500	40 ± *5.9*	17 ± *7.5*	2 ± *0.0*	59 ± *12.0*	**24**
600	30 ± *8.9*	15 ± *4.8*	0 ± *0.0*	45 ± *13.6*	**18**
800	30 ± *7.2*	20 ± *2.6*	0 ± *0.0*	50 ± *9.2*	**20**
1000	34 ± *0.6*	19 ± *1.5*	0 ± *0.0*	53 ± *1.9*	**21**

Table 3.3 *Amount of progeny produced over a range of $CuSO_4$ concentrations. Results shown as average daily brood size, over 72 hours, total brood size and as a percentage of control, ± SEM (n=6).*

The number of progeny produced over a range of $CuSO_4$ concentrations was determined every 24 hours over a 3 day period ***Figure 3.3***.

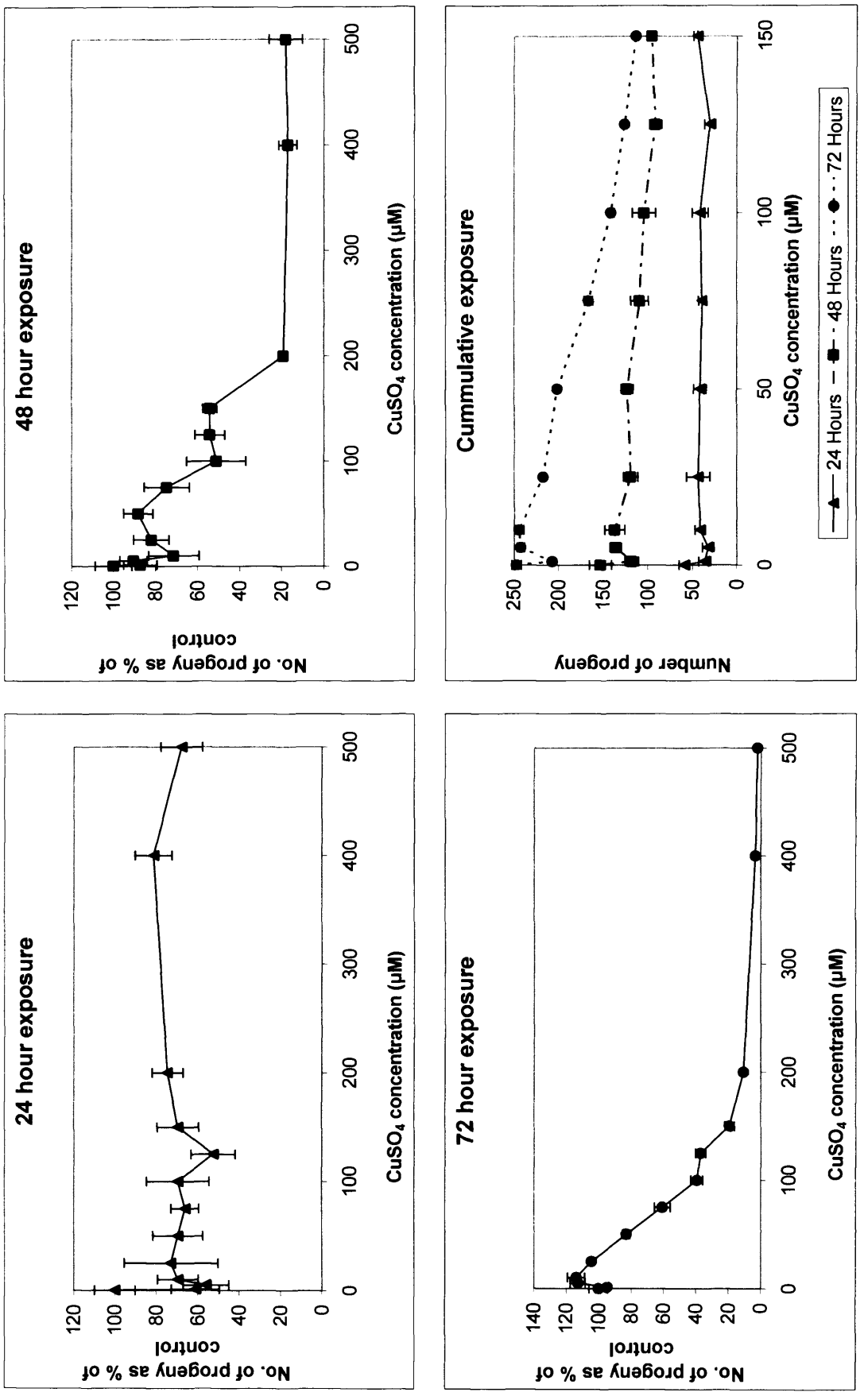

Figure 3.3 *The number of progeny produced every 24 hours and the cumulative number of progeny over a 3 day period, ± SEM (n=6).*

The $CuSO_4$ concentration had little effect on *C. elegans* over 24 hours but a substantial effect after 72 hours (***Figure 3.3***). The EC50 of $CuSO_4$ was chosen to be calculated over 3 days as this is the minimum time period needed for experiments with *C. elegans*, allowing for progeny to be produced and develop through a complete life cycle. ***Figure 3.4*** shows the EC50 of Cu in *C. elegans* over a 3 day period by measuring total brood size over a range of $CuSO_4$ concentrations.

There appears to be a slight beneficial effect at the lower Cu concentrations before the number of progeny rapidly decreases as the Cu concentration increases. The nominal EC50 of $CuSO_4$ in *C. elegans* over a period of 3 days was found to be 100μM, which equates to 70μM of nitric acid extractable Cu (***Table A.1***).

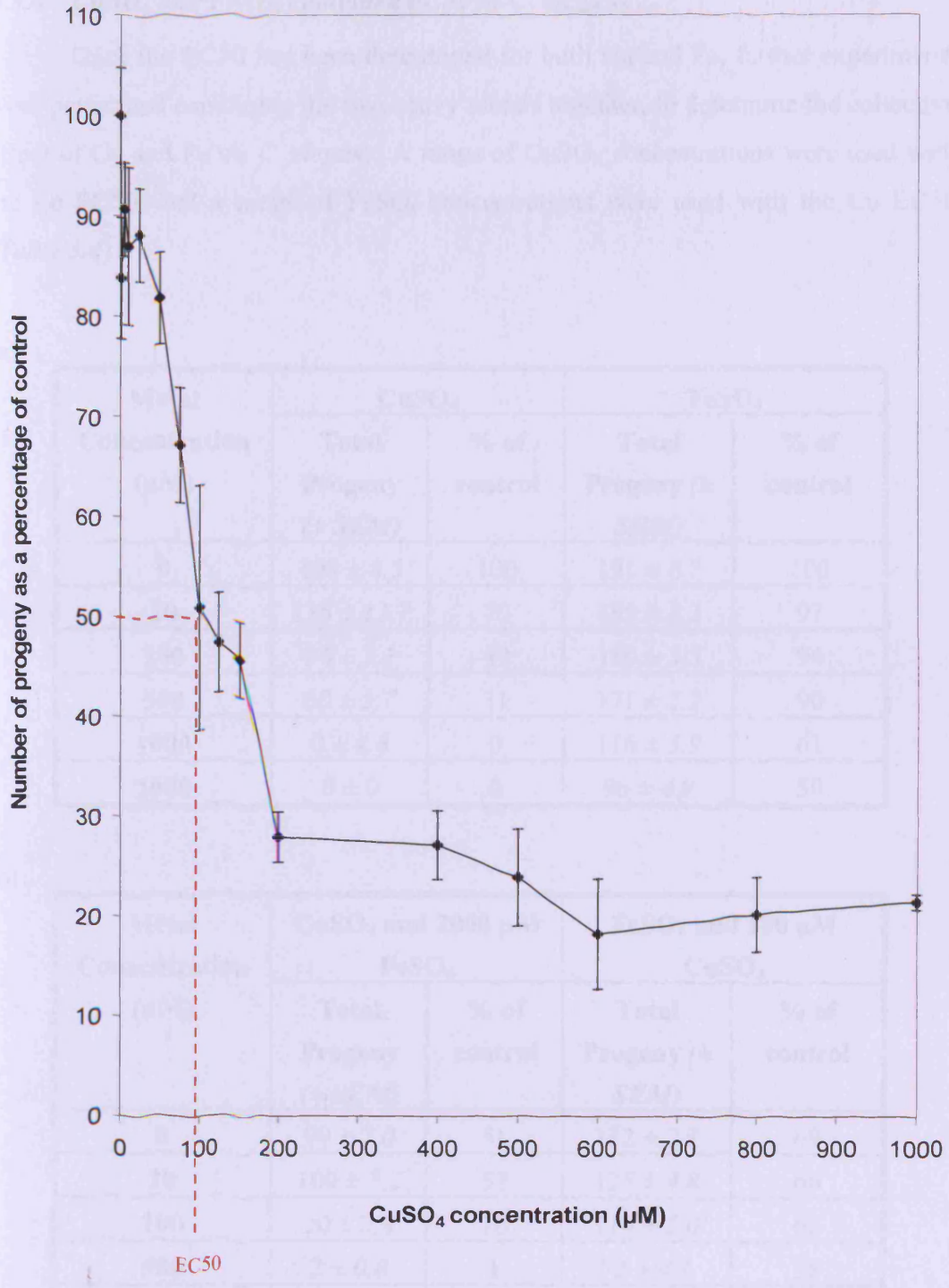

Figure 3.4 *$CuSO_4$ EC50 in* C. elegans. *Effect of a range of $CuSO_4$ concentrations on the total brood size over a 3 day period, with the EC50 indicated, ± SEM (n=6).*

3.3.4 *$CuSO_4$ and $FeSO_4$ combined EC50 in* C. elegans

Once the EC50 had been determined for both Cu and Fe, further experiments were performed combining the two heavy metals together, to determine the collective effect of Cu and Fe on *C. elegans*. A range of $CuSO_4$ concentrations were used with the Fe EC50, and a range of $FeSO_4$ concentrations were used with the Cu EC50 (***Table 3.4***).

(a)

Metal Concentration (µM)	$CuSO_4$		$FeSO_4$	
	Total Progeny (± *SEM*)	% of control	Total Progeny (± *SEM*)	% of control
0	193 ± *8.3*	100	191 ± *8.7*	100
20	136 ± *11.7*	70	185 ± *5.2*	97
100	94 ± *3.1*	49	180 ± *3.5*	94
500	60 ± *5.7*	31	171 ± *5.2*	90
1000	0 ± *4.8*	0	116 ± *3.9*	61
2000	0 ± *0*	0	96 ± *4.9*	50

(b)

Metal Concentration (µM)	$CuSO_4$ and 2000 µM $FeSO_4$		$FeSO_4$ and 100 µM $CuSO_4$	
	Total Progeny (± *SEM*)	% of control	Total Progeny (± *SEM*)	% of control
0	99 ± *7.0*	51	132 ± *2.7*	69
20	100 ± *5.2*	52	125 ± *4.8*	66
100	20 ± *2.4*	10	119 ± *5.0*	62
500	2 ± *0.8*	1	72 ± *4.1*	38
1000	0 ± *0.0*	0	65 ± *9.7*	34
2000	0 ± *0.0*	0	60 ± *2.7*	31

Table 3.4 *Heavy metal effects on* C. elegans *with (a) showing individual Cu and Fe EC50 results and (b) showing the combined Cu and Fe EC50 result. Results shown as total progeny products and as a percentage of control, ± SEM (n=6).*

The $CuSO_4$ EC50 in *C. elegans* over a 3 day period was 100μM, whilst the $FeSO_4$ EC50 was 2000μM. The graph below (***Figure 3.5***) shows the affect of combining Cu and Fe in NGM agar plates on the number of progeny produced by *C. elegans*.

Table 3.4 and the graph in ***Figure 3.5*** shows that $CuSO_4$ is more toxic then $FeSO_4$ to *C. elegans* and the combination of both metals increases the detrimental effect on the nematodes in a synergistic manner.

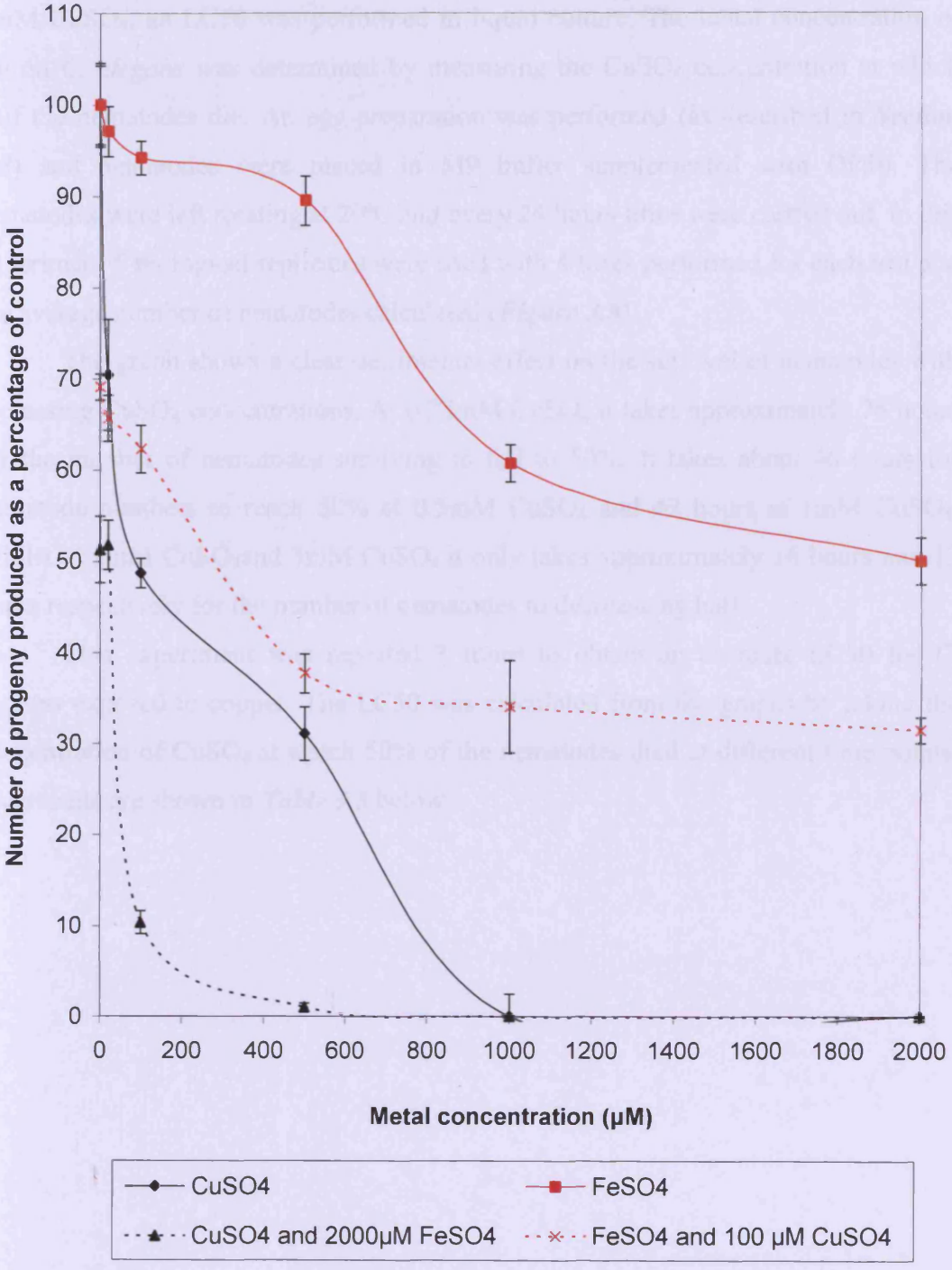

Figure 3.5 *$CuSO_4$ and $FeSO_4$ combined EC50 results. Showing the total number of progeny produced as a percentage of the control, ± SEM (n=6).*

3.3.5 *$CuSO_4$ LC50 of* C. elegans *in liquid culture*

As the *E. coli* strain OP50 does not grow on agar plates containing more then 1mM $CuSO_4$, an LC50 was performed in liquid culture. The lethal concentration of Cu on *C. elegans* was determined by measuring the $CuSO_4$ concentration at which half the nematodes die. An egg preparation was performed (as described in ***Section 2.3***) and nematodes were placed in M9 buffer supplemented with OP50. The nematodes were left rotating at 20°C and every 24 hours titres were carried out. In this experiment 5 biological replicates were used with 4 titres performed for each test and the average number of nematodes calculated (***Figure 3.6***).

The graph shows a clear detrimental effect on the survival of nematodes with increasing $CuSO_4$ concentrations. At 0.25mM $CuSO_4$ it takes approximately 76 hours for the number of nematodes surviving to fall to 50%. It takes about 46 hours for nematode numbers to reach 50% at 0.5mM $CuSO_4$ and 42 hours at 1mM $CuSO_4$. Whilst at 2mM $CuSO_4$and 3mM $CuSO_4$ it only takes approximately 16 hours and 13 hours respectively for the number of nematodes to decrease by half.

This experiment was repeated 3 times to obtain an accurate LC50 for *C. elegans* exposed to copper. The LC50 was calculated from the graphs by taking the concentration of $CuSO_4$ at which 50% of the nematodes died at different time points. The results are shown in ***Table 3.5*** below.

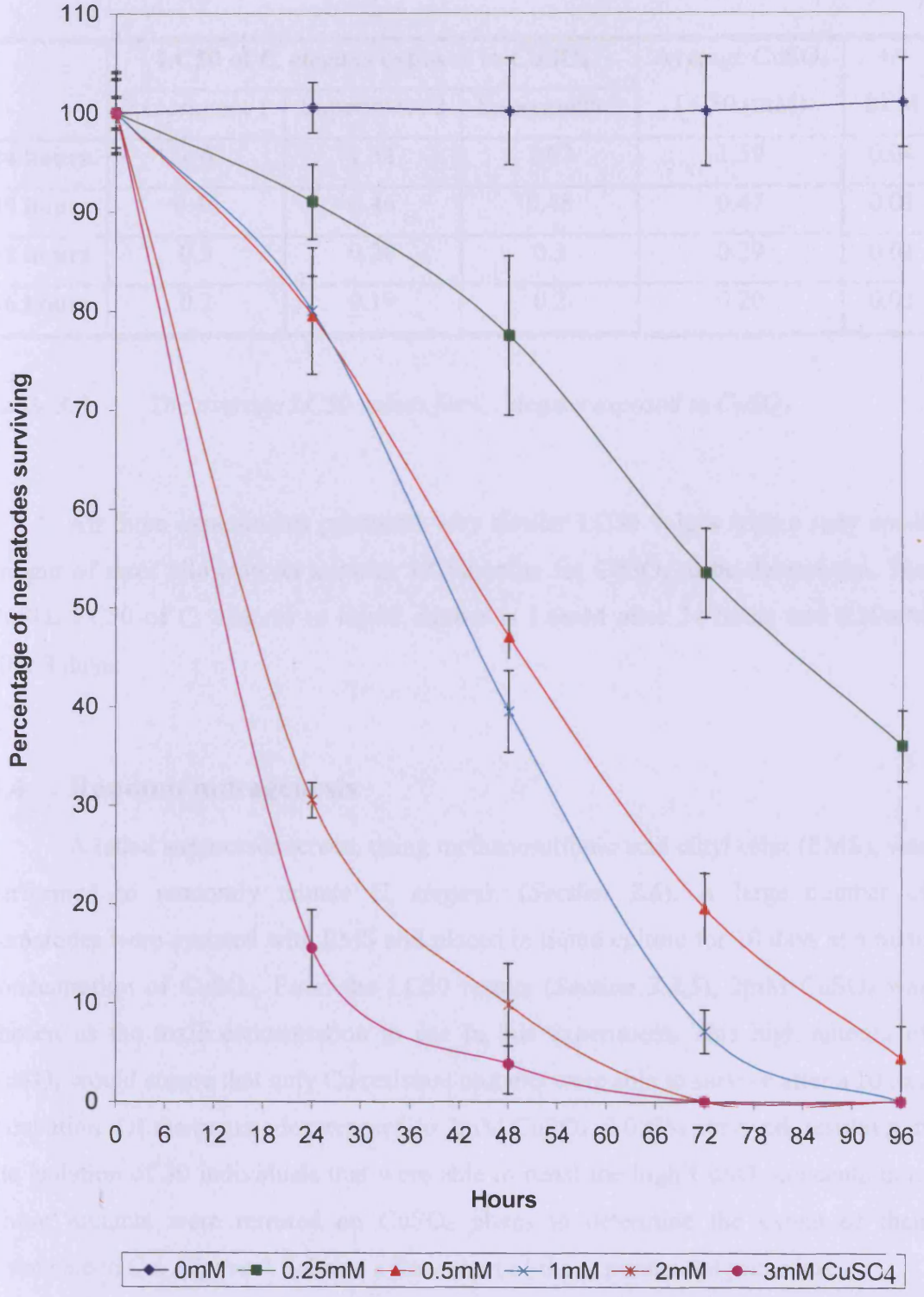

Figure 3.6 *The effect of $CuSO_4$ on the number of nematodes in liquid culture. Results shown as the percentage of nematodes surviving over 4 days at different $CuSO_4$ concentrations, ± SEM (n=5).*

	LC50 of *C. elegans* exposed to $CuSO_4$			Average $CuSO_4$ LC50 (mM)	+/- SEM
	Experiment 1	Experiment 2	Experiment 3		
24 hours	1.6	1.54	1.62	1.59	0.04
48 hours	0.46	0.46	0.48	0.47	0.01
72 hours	0.3	0.28	0.3	0.29	0.01
96 hours	0.2	0.19	0.2	0.20	0.01

Table 3.5 *The average LC50 values for* C. elegans *exposed to* $CuSO_4$

All three experiments produced very similar LC50 values with a very small margin of error allowing an accurate LC50 value for $CuSO_4$ to be determined. The $CuSO_4$ LC50 of *C. elegans* in liquid culture is 1.6mM after 24 hours and 0.29mM after 3 days.

3.4 Random mutagenesis

A lethal suppressor screen, using methanesulfonic acid ethyl ester (EMS), was performed to randomly mutate *C. elegans,* (***Section 2.6***). A large number of nematodes were mutated with EMS and placed in liquid culture for 10 days at a toxic concentration of $CuSO_4$. From the LC50 results (***Section 3.3.5***), 2mM $CuSO_4$ was chosen as the toxic concentration to use in this experiment. This high amount of $CuSO_4$ would ensure that only Cu resistant mutants were able to survive after a 10 day incubation. Of the nematodes exposed to 2mM $CuSO_4$, 0.003% survived, resulting in the isolation of 30 individuals that were able to resist the high $CuSO_4$ concentration. These mutants were retested on $CuSO_4$ plates to determine the extent of their resistance to Cu. ***Figure 3.7*** shows a flow chart of the experimental procedure.

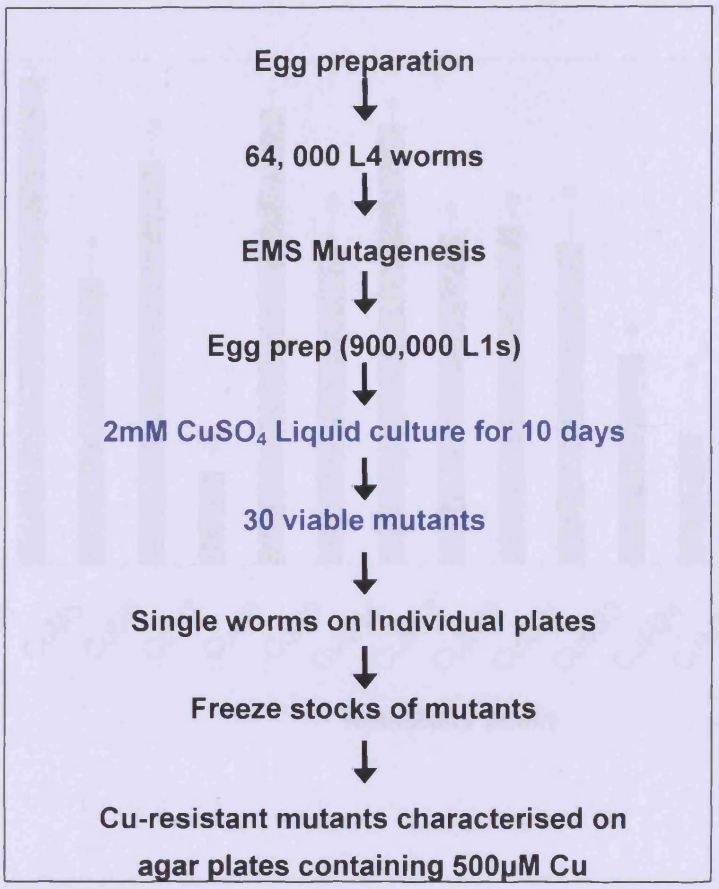

Figure 3.7 *Experimental design of the lethal suppressor screen by random mutagenesis.*

Out of the 900,000 nematodes exposed to a lethal concentration of $CuSO_4$ after treatment with EMS, 30 nematodes survived and were placed onto individual agar plates. Of the 30 survivors (designated CuM1-30) only 14 produced viable offspring. These were maintained on agar plates and when numbers reached an appropriate level, stocks were frozen away for future use (***Section 2.2.3***). The 14 mutants were assessed for their resistance to Cu by determining the brood size at a Cu concentration of 500µM. For each mutant strain, six nematodes were placed on individual agar plates and the number of progeny was determined every 24 hours with the adult being removed onto a fresh plate at appropriate intervals. As a control the brood size of wild-type nematodes (N2 strain) was analysed in parallel. ***Figure 3.8*** shows the total number of progeny produced by each mutant compared to the wild-type strain.

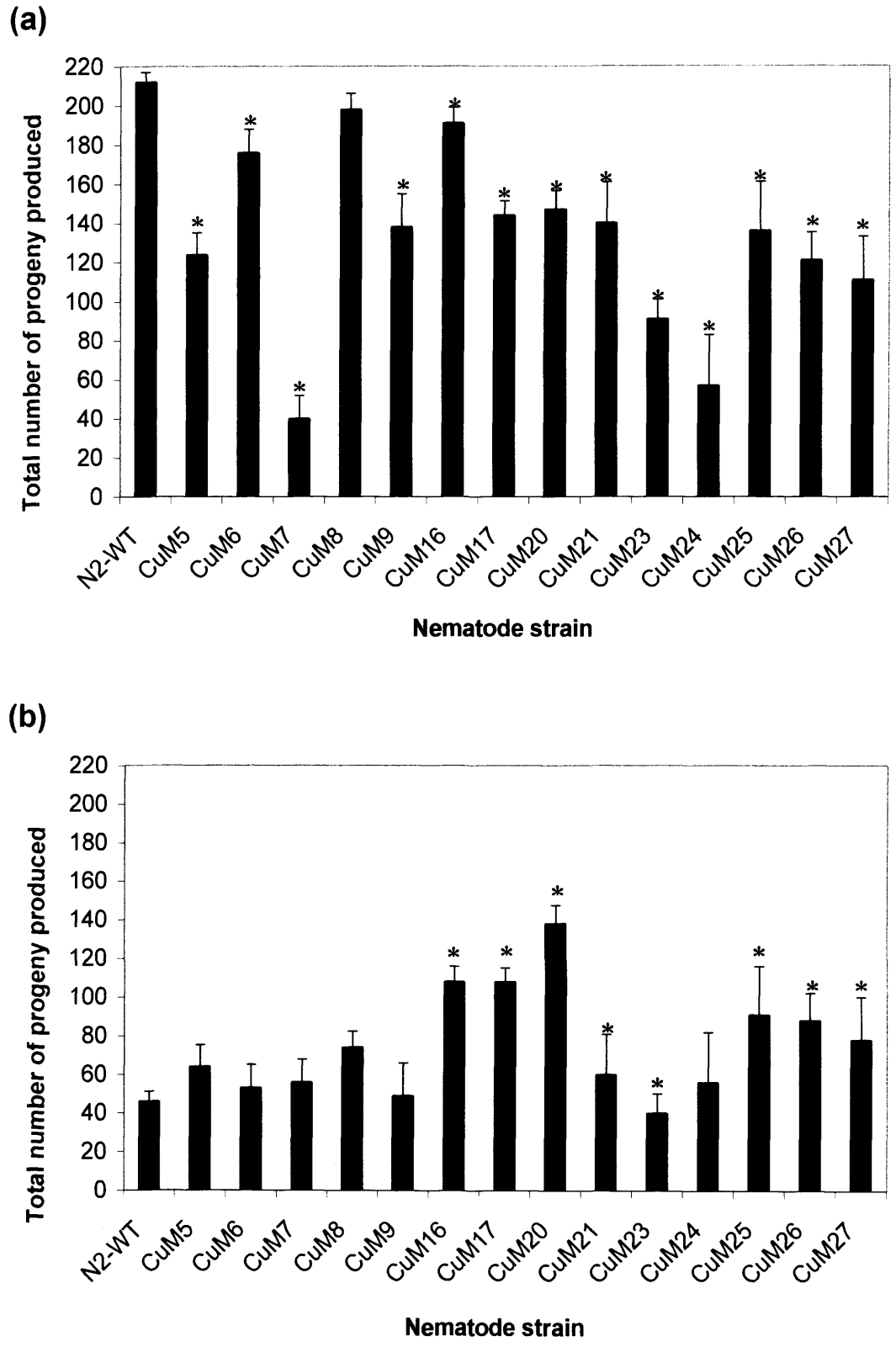

Figure 3.8 *The total number of progeny produced by each* C. elegans *strain on(a) 0μM and (b) 500μM $CuSO_4$, ± SEM (n=6).*
** denotes a significant difference from the N2-WT strain.*

Based on an Anderson-Darling plot, all results are normally distributed; hence two-sample t-tests were performed to determine the significant difference between the means. A statistical significant difference was identified by a p-value of less than 0.05. ***Figure 3.8 (a)*** shows that on control agar plates the wild-type nematode strain N2 produces 212 progeny whist all EMS mutated strains except CuM8 produced significantly less offspring than the N2 strain. In particular strains CuM7, CuM23 and CuM24 produce only 40, 91 and 57 progeny respectively on 0μM $CuSO_4$.

Figure 3.8 (b) shows that on 500μM $CuSO_4$ the wild-type nematode strain N2 produces only 46 offspring. Out of the 14 viable CuM strains isolated from the EMS exposure, 7 (CuM16, CuM17, CuM20, CuM21 CuM25, CuM26 and CuM27) produce significantly more offspring on 500μM $CuSO_4$ than the wild-type strain indicating varying degrees of resistance to Cu. With CuM16, CuM17 and CuM22 producing as many as 108, 108, and 138 progeny respectively. Strain CuM23 produces significantly less progeny than wild-type nematodes on 500μM $CuSO_4$.

Figure 3.9 shows the number of progeny produced by each *C. elegans* mutant at 0μM and 500μM $CuSO_4$ as a percentage of the number of offspring produced by the control N2 strain.

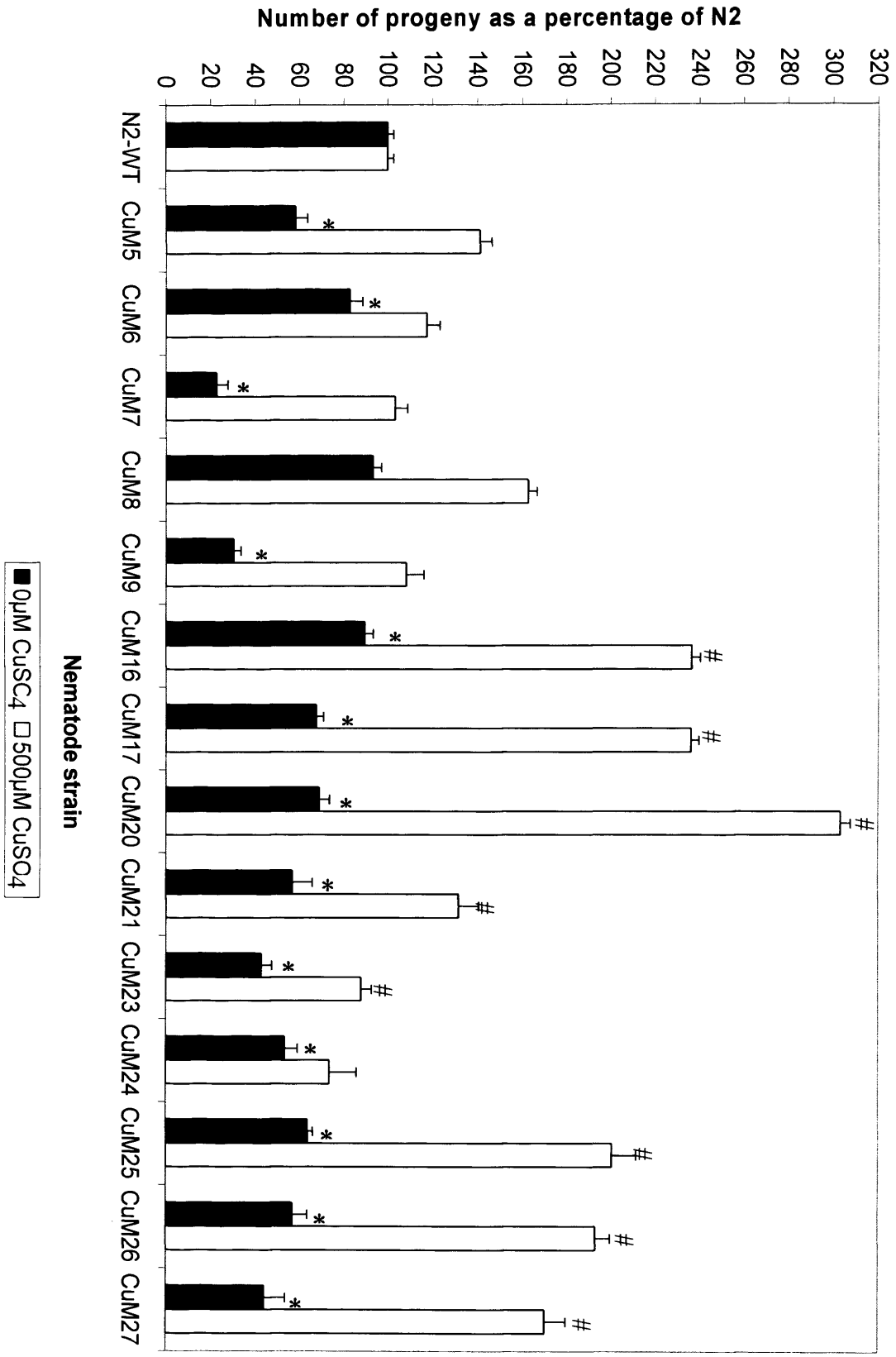

Figure 3.9 *Number of progeny produced by each* C. elegans *mutant on 0μM and 500μM $CuSO_4$ as a percentage of the control N2 strain, ± SEM (n=6). * denotes a significant difference from the N2-WT strain on* 0μM $CuSO_4$. # *denotes a significant difference from the N2-WT strain on 500μM $CuSO_4$.*

The graph in ***Figure 3.9*** shows that each EMS mutated nematode strain was affected differently by $CuSO_4$ compared to the wild-type N2 stain in terms of the number of progeny produced. Remarkably 4 strains (CuM16, CuM17, CuM20 and CuM25) produce at least 100% more offspring on agar containing 500µM $CuSO_4$ than the wild-type nematodes, with the CuM20 strain producing over 3-fold more offspring on Cu plates compared to control. Conversely all strains except CuM8 produced significantly less progeny on control plates compared to the wild type strain, ranging from a decrease of 7% (CuM8) to 77% (CuM7).

Table 3.6 shows the percentage reduction in progeny from 0µM $CuSO_4$ to 500µM $CuSO_4$ for N2 and all EMS mutated strains.

Nematode strain	Percentage reduction in progeny from 0µM $CuSO_4$ to 500µM $CuSO_4$	2-sample T-Test P-values
N2-WT	- 79 %	0.000*
CuM5	- 48 %	0.003*
CuM6	- 70 %	0.000*
CuM7	- 3 %	0.671
CuM8	- 63 %	0.000*
CuM9	- 24 %	0.973
CuM16	- 44 %	0.001*
CuM17	- 25 %	0.003*
CuM20	- 6 %	0.652
CuM21	- 50 %	0.023*
CuM23	- 56 %	0.018*
CuM24	- 71 %	0.364
CuM25	- 33 %	0.472
CuM26	- 27 %	0.127
CuM27	- 17 %	0.518

Table 3.6 *Percentage difference in number of progeny produced by EMS treated nematodes compared to N2, and on 500µM $CuSO_4$ compared to 0µM $CuSO_4$, ± SEM (n=6). * Denotes a significant difference (a p-value of less than 0.05) in the number of progeny produced on 500µM $CuSO_4$ compared to 500µM $CuSO_4$.*

Table 3.6 shows that in all CuM strains there is less of a reduction in the number of progeny produced on 500μM $CuSO_4$ compared to 0μM $CuSO_4$ than the wild-type strain. The N2 wild type strain produces 79% less progeny on agar plates containing 500μM $CuSO_4$ compared to agar with 0μM $CuSO_4$. Importantly, with the EMS mutated strains CuM7, CuM9, CuM20, CuM24, CuM25, CuM26 and CuM27 there is no significant reduction in the number of progeny produced on 500μM $CuSO_4$ compared to 0μM $CuSO_4$, indicating some resistance to Cu toxicity. With CuM7 and CuM20 having only a 3% and 6% reduction respectively, in the number of progeny produced on 500μM $CuSO_4$ compared to 0μM $CuSO_4$.

3.5 Discussion

The AAS results show that the nitric acid extractable amount of Cu is significantly less compared to the nominal amount of $CuSO_4$ added to the NGM agar plates. This may be due to oxidation of the $CuSO_4.7H_2O$ crystals prior to dissolving in water. The AAS results determined that NGM agar has basal amounts of 6μM Cu and 70μM Fe. There was no significant difference in the Cu concentration with and without adding $CuSO_4$ to the OP50 culture before streaking onto the plates. This confirmed that $CuSO_4$ could be added to the bacteria before spreading onto the agar, to ensure that the nematodes were fully exposed to the Cu, without resulting in any significant increase in the amount of Cu present.

On NGM agar plates the average amount of progeny produced by nematodes is 200-300 in their life time. There appears to be a slight beneficial effect at the lower Cu concentrations before the number of progeny rapidly decreases as the Cu concentration increases ***(Figure 3.3 and 3.4)***. This is known as the hormesis effect, which is a dose response phenomenon characterized by a low dose stimulation and high dose inhibition. It may be primarily an adaptive response to stress where lower levels of Cu are not harmful, but may indeed be beneficial. In these studies, a low concentration of 20μM $CuSO_4$ sometimes had beneficial effects on the nematodes while concentrations above 100μM had detrimental consequences to the nematodes. This hormetic effect is not unexpected as Cu is an essential metal at low concentrations but toxic at high concentrations.

The EC50 of $CuSO_4$ in *C. elegans* over a period of 3 days was found to be

0.1mM, which equates to 0.07mM of nitric acid extractable Cu by AAS. While in liquid culture the $CuSO_4$ LC50 of *C. elegans* was found to be 1.59mM over 24 hours and 0.29mM after 3 days. Cu and Fe homeostasis are intertwined and Fe in the environment may increase the toxicity of Cu, thus the EC50 of $FeSO_4$ in *C. elegans* was determined and found to be 2mM over a period of 3 days. As the basal level of Fe present in the NGM agar was only 0.07mM and the EC50 was 2mM, the background level of Fe was not at a concentration that would significantly interfere with the Cu toxicity experiments.

The addition of Fe in the Cu toxicity experiments caused the number of progeny to be reduced in a synergistic manner (***Figure 3.5***). Thus the combination of both heavy metals together increases the toxicity. The effect is synergistic rather than additive showing that homeostasis of the two heavy metals are linked (***Figure 3.2***). This is largely due to the role of Cu as a cofactor for the ferroxidase Fet3, which imports Fe into the cell and may also act in the cellular defence against Cu toxicity.

EMS was used to chemically mutate the *C. elegans* DNA to induce Cu resistance. The experiment resulted in 14 viable mutants which displayed varying degrees of resistance to Cu, 9 of which produced significantly more offspring on agar plates containing 500μM $CuSO_4$ than the N2 control. Importantly the EMS mutated strains CuM7, CuM9, CuM20, CuM24, CuM25, CuM26 and CuM27 showed no significant reduction in the number of progeny produced on 500μM $CuSO_4$ compared to 0μM $CuSO_4$ (***Table 3.6***). This indicates that these strains have some resistance to Cu toxicity in terms of the number of progeny produced.

Interestingly all Cu resistant mutants produced significantly less progeny than the wild-type strain on control plates. This may be a compensation effect where the mutation giving rise to Cu resistance is allowing them to produce more progeny at toxic levels of Cu, which is compensated for by less progeny on control plates due to the fitness cost.

The differences between N2 and the EMS treated nematodes indicated that there are numerous genes involved in Cu homeostasis which is normally tightly controlled by many components. For instance, a mutation in the Cu importer Ctr, would result in an impaired ability to import Cu into the cell. This would lead to the nematode being able to perform better at higher Cu concentrations, whilst having detrimental consequences where there is a limited availability of Cu. This correlates with the results from the random mutagenesis in that more progeny are produced on

500μM $CuSO_4$, whilst less progeny are produced on 0μM $CuSO_4$ compared to the wild-type strain.

At toxic concentrations of $CuSO_4$ (100μM and above), detrimental effects are seen in *C. elegans* with a reduced brood size and developmental problems. The nematodes on $CuSO_4$ plates are always smaller, exhibiting reduced development, slower movement and with various defects observed. These initial experiments were carried out to determine the whole organism response of *C. elegans* to $CuSO_4$. The EC50 and LC50 of $CuSO_4$ in *C. elegans* were calculated, thus further studies were performed at appropriate concentrations of $CuSO_4$.

The Ctr Cu importer shown in ***Figure 3.2*** is from a widely conserved family of high-affinity Cu transport proteins that mediates Cu uptake at the plasma membrane. In this study a putative *C. elegans* Ctr will be investigated by reverse genetics. As Cu homeostasis is a complex process controlled by many different proteins it was necessary to examine more than a single gene. The Ctr gene has been putatively identified as encoding a Cu importer, so a second gene involved in a contrasting aspect of Cu metabolism was sought for further evaluation. For comparison a putative cytosolic chaperone encoding gene (CutC), implicated in Cu efflux from the cell, was chosen for investigation using the RNAi system within the *C. elegans* model organism.

Chapter 4

Bioinformatic analysis of the putative copper transporters Ctr and CutC

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4.1 Introduction

The aim of this study was to determine the expression profile and functional significance of two putative Cu transporters in *C. elegans,* Ctr and CutC. Ctr is membrane-bound Cu transporter, that is well characterised in many species (***Figure 1.4***). Whilst sequence analysis of the CutC gene suggests that it is a small cytoplasmic Cu-binding protein. CutC has been well characterised in *E. coli* and identified in a wide range of different species including humans. In *E. coli*, the CutC gene is postulated to code for an efflux protein which removes excess Cu from the cytoplasm (Gupta *et al.*, 1995).

Bioinformatic analysis was performed on the *C. elegans* Ctr and CutC proteins to infer as much information as possible about the putative Cu transporters. Cu binding motifs were identified in the *C. elegans* Ctr and several bioinformatic tests were performed to determine the probability of either being a membrane bound protein and to predict their topology. QPCR was utilised to determine if the expression level of Ctr and CutC was up or down regulated with exposure to $CuSO_4$. Finally the Ctr and CutC gene were knocked-down by RNAi to determine their functional significance in *C. elegans*. The effect of Cu on the life cycle parameters of *C. elegans* was fully characterised in this study with and without RNAi of Ctr and CutC.

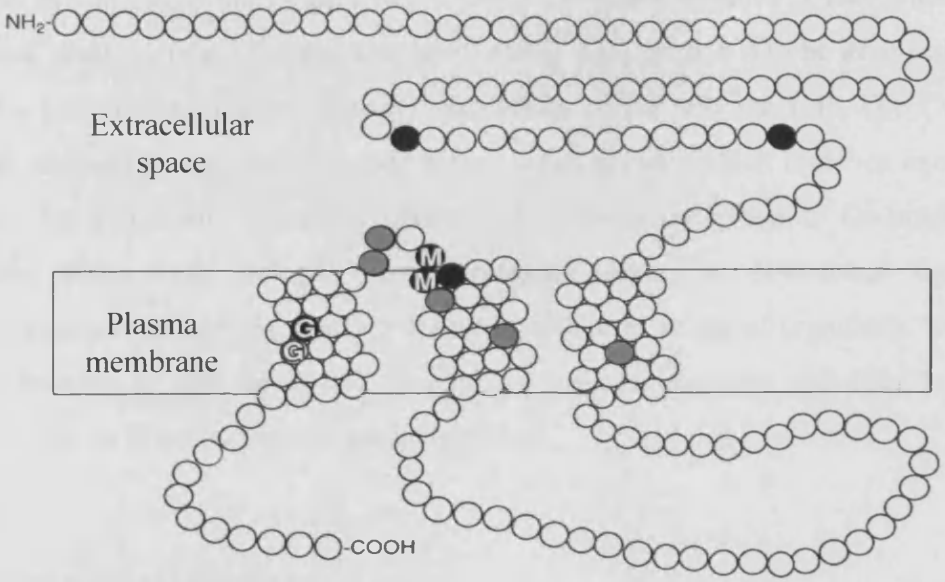
4.1.1 Background to Ctr

The Ctr family consists of integral membrane proteins that are located on the plasma membrane, as well as on intracellular vesicular membranes (***Figure 4.1***). Initial uptake of the essential heavy metal Cu into cells occurs through the Ctr family of proteins, which function in organisms as diverse as yeast, plants and metazoans, including mice and humans (Aller *et al.*, 2004).

Ctr has been studied extensively in yeast, yet the putative Ctr in *C. elegans* remains uncharacterised. Thus the purpose of this study was to identify Ctr and determine its expression profile and functional significance in *C. elegans*. Initially the putative Ctr in *C. elegans* was found by BLAST searches, which were validated by looking for the characteristics found in all Ctr family members.

The amino acid (AA) sequence is poorly conserved between members of the Ctr family and only two sequence motifs, Met-*X*-*X*-*X*-Met (MXXXM) and Gly-*X*-*X*-*X*-Gly (GG4) in the second and third transmembrane (TM) domains, respectively, are invariantly conserved within all of the Ctrs (***Figure 4.1***). Based on the AA sequence of Ctr proteins they are predicted to have three transmembrane α-helices and lack nucleotide binding domains. Previous biochemical characterisation of Ctr proteins shows that they form oligomers in the membrane and that the N-terminal is located in the extracellular space.

A



B

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                                    TM3
A. thaliana COPT1                   SFNAGVFLVALAGHAVGFMLFGSQTF
C. albicans CTR1                    TYTLTYFFAVVIGSGVGRFVAERLME
C. elegans CE03429                  VFSVYICLSLCFGLAIGHFVFASRTG
C. elegans CE26194                  TFNAYLCFFTVVGEVVCHLLYRTLYP
D. melanogaster CTR1A               TYNVWLCLMVVLGAAVGYFLFCWKKS
D. melanogaster CTR1B               TFNYWLCLAVILGLGLGYFFFGWNKK
D. melanogaster CTR1C               TFNVWLCVAVLLGAGVGYYIFCAFRT
D. rerio CTR1                       TYNGYLCIAVAAGAGLGYFLFSWKKA
H. sapiens CTR1                     TYNGYLCIAVAAGAGTGYFLFSWKKA
H. sapiens CTR2                     SYNTWIFLGVVLGSAVGYYLAYPLLS
M. musculus CTR1                    TYNGYLCIAVAAGAGTGYFLFSWKKA
M. musculus CTR2                    SYNTWIFLGVVLGSAVGYYLAYPLLN
P. sicula CTR1                      TYNGYLCIAVAAGAGTGYFLFSWKKA
R. norvegicus CTR1                  TYNGYLCIAVAAGAGTGYFLFSWKKA
S. cerevisiae CTR1                  SFVLTYVFAVITGLALSEVFFNRCKI
S. cerevisiae CTR2                  TYNGWLMLAVVCGAIWGNYSWCTSYS
S. cerevisiae CTR3                  YYNGYIIISCLIGAIVGRFIFCYEPL
S. pombe CTR2                       TYNAYVILAIAIGAAFGYRRSHCDTV
S. pombe CTR4                       YYNGYVILFLFCGTFFGYFLFGADTI
S. pombe CTR5                       SFNGYAILFGFVGAWIGFFLFASDTY
S. pombe CTR6                       TYNAYVILAIAIGAAFGYRRSHCDTV
S. scrofa CTR1                      TYNGYLCIAVAAGAGTGYFLFSWKKA
X. laevis CTR                       SYNAAIFIAVILGSGLGYFLAFPLLS
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Figure 4.1 ***(a)*** *Topology of the human Ctr monomer showing the extracellular N terminus, three putative TM domains and an intracellular C terminus. The motif MXXM in the second TM domain and GG4 in the third TM domain are indicated and found in the entire family of Ctrs. AAs represented as black circles are invariantly conserved in Ctrs from yeast, plants and metazoans, and those in gray are >90% identical in the family.* ***(b)*** *Multiple AA sequence alignment of the third TM domain reveals a nearly invariant conservation of the GG4 motif in all of the known members of the Ctr family (adapted from Aller* et al., *2004).*

4.1.2 Background to CutC

In *E. coli* preliminary characterisation of Cu-sensitive mutants identified 6 genes (*cutA*, *cutB*, *cutC*, *cutD*, *cutE* and *cutF*) which were proposed to be involved in the uptake, intracellular storage, delivery and efflux of Cu (***Figure 1.5***). The CutC gene in *E. coli* was postulated to encode for an efflux protein which removes excess Cu from the cytoplasm. CutC is characterised as a cytoplasmic Cu-binding hydrophilic protein with multiple charged residues lacking an N-terminal signal sequence (Gupta *et al.*, 1995). CutC is found in a diverse range of organisms from mouse to humans as well as *E. coli*, (though no orthologues were identified in *S. cerevisiae*), yet its function remains uncharacterised.

4.2 Sequence alignments

4.2.1 Sequence alignment of Ctr

The Ctr protein has been identified and well characterised in a wide range of different organisms with a number of different proteins representing different affinity membrane bound Cu transporters in the Ctr family. *C. elegans* contains at least 7 Ctr proteins, of which only 3 have been confirmed as Cu transporters. In this study the gene encoding the putative Ctr Cu transporter (Wormbase ID: F58G6.3) was identified in *C. elegans* by BLAST searching (using NCBI and the Wormbase database). ***Figure 4.2*** shows the AA alignment of F58G6.3 with other Ctr proteins in *C. elegans*. The alignment shows that though the AA sequence is poorly conserved between different members of the Ctr family in *C. elegans*, they all contain the putative Cu binding motif MXXXM and four of them have the conserved GG4 motif.

F58G6.3 shows homology with various members of the Ctr family from different species. Thus it is not known which family member F58G6.3 belongs to or whether it is a high or low affinity Cu uptake transporter. ***Figure 4.3*** shows the AA alignment of the *C. elegans Ctr* with various species.

This alignment again shows that there is little AA sequence homology with Ctr from different species, but highlights two putative Cu binding motifs and a GG4 motif which are invariantly conserved within all of the Ctrs.

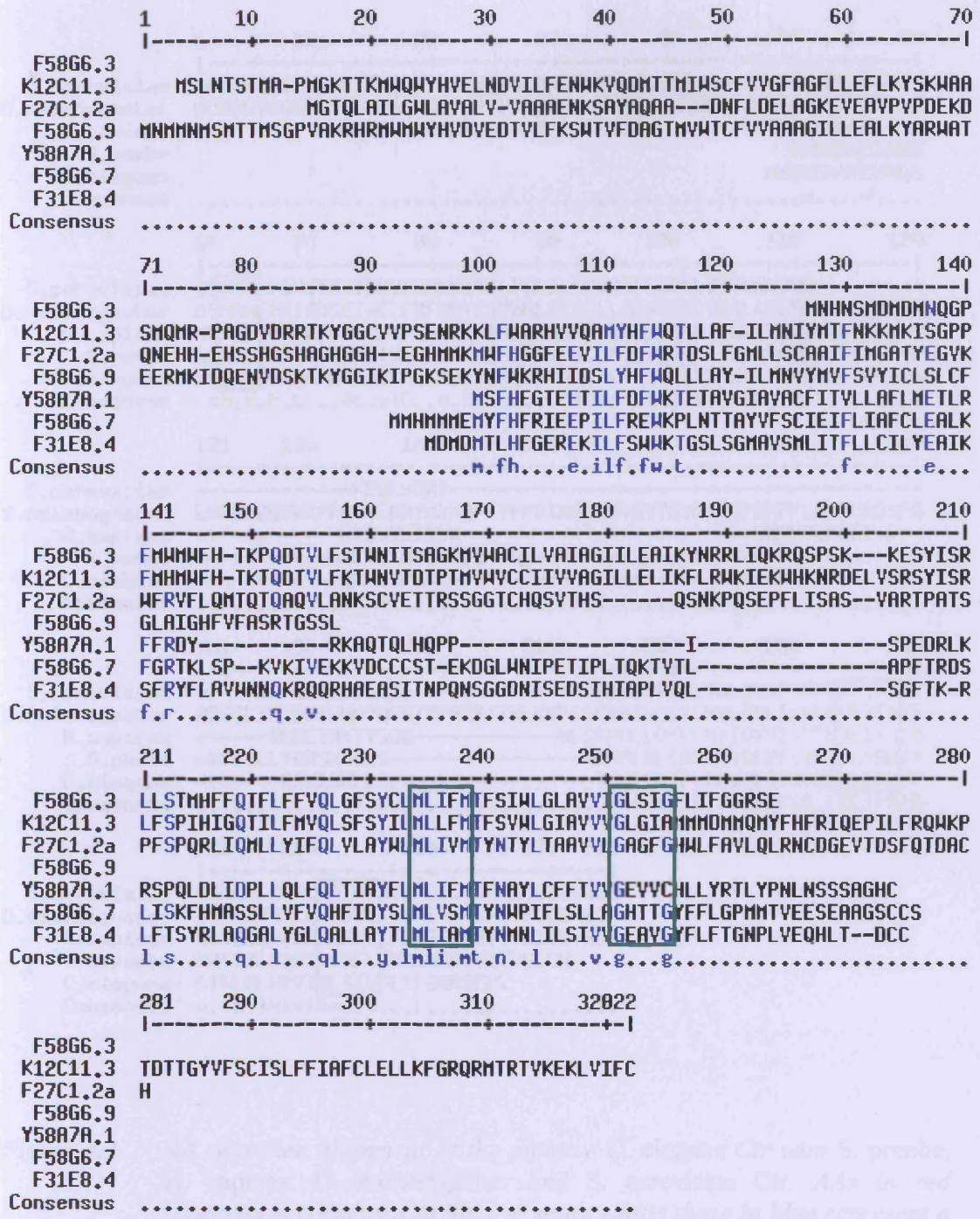


Figure 4.2 *AA sequence alignment of the putative Ctrs in* C. elegans. *AAs in blue shows consensus of 50% or more. The MXXXM and GG4 Cu binding motifs are indicated with a green box. The alignment was performed using Multiple sequence alignment with hierarchical clustering (Corptet, 1988).*

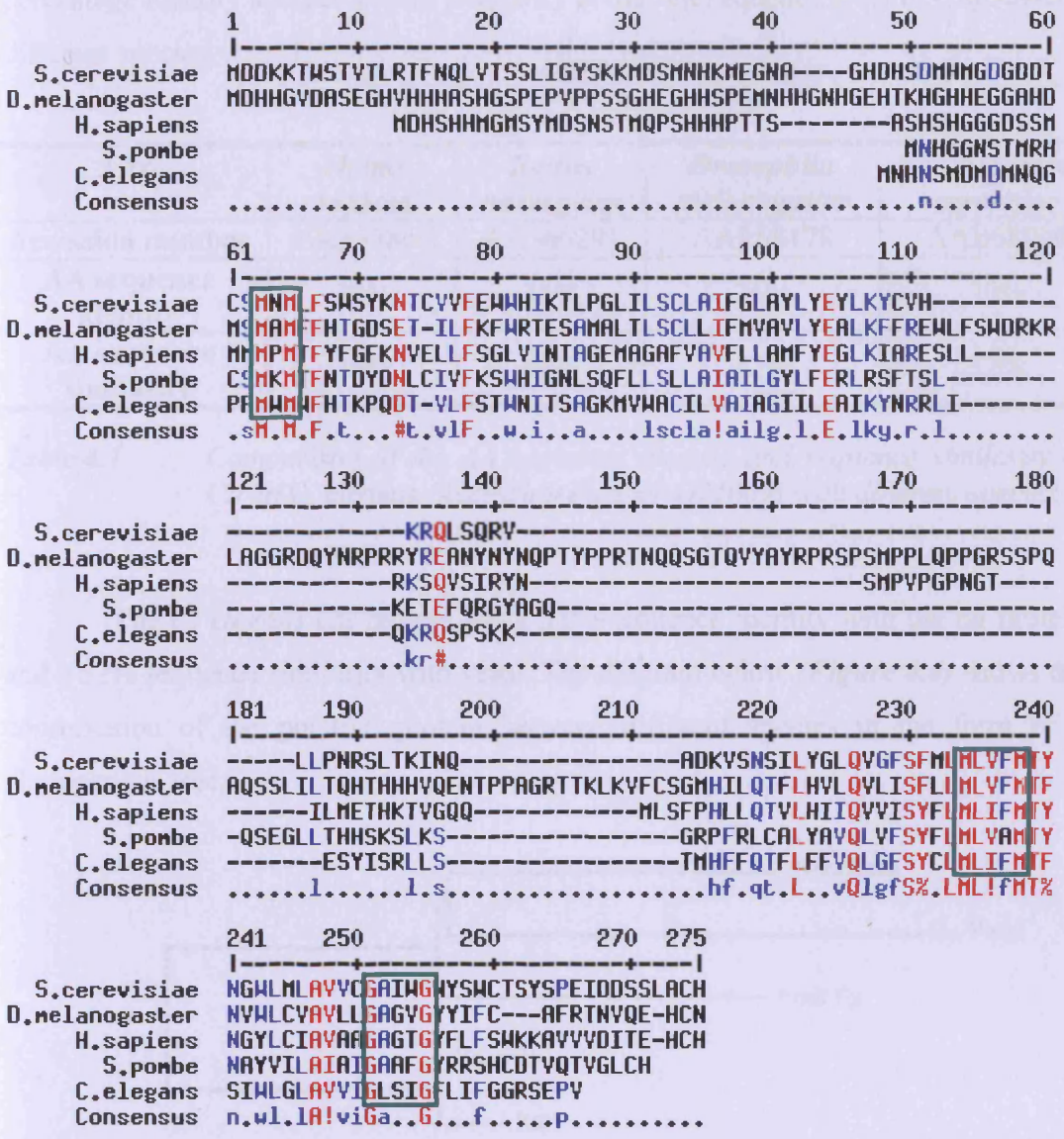


Figure 4.3 *AA sequence alignment of the putative* C. elegans *Ctr with* S. pombe, H. sapiens, D. melanogaster, *and* S. cerevisiae Ctr. *AAs in red represent a consensus of 90% or more whilst those in blue represent a consensus of 50% or more. In the consensus sequences symbols indicate AAs of similar properties; % represents aromatic AAs, # represents polar AAs and ! represents C-beta branched aliphatic AAs. The putative MXM and MXXXM Cu binding motifs and GG4 motif are indicated by a green box. The alignment was performed using multiple sequence alignment with hierarchical clustering (Corpet, 1988).*

Table 4.1 below shows the results of the BLAST search giving the percentage identity and percentage similarity at the AA sequence level of Ctr between different species.

Ctr	*Homo sapiens*	*Rattus norvegicus*	*Drosophila melanogaster*	*Saccharomyces cerevisiae*
Accession number	AK333866	AA566291	AAL68178	AAB68020
AA sequence identity	29%	31%	26%	30%
AA sequence similarity	46%	47%	42%	52%

Table 4.1 *Comparison of the AA sequence identity and sequence similarity of Ctr in* C. elegans *(Accession number Q21009) with different species.*

The *C. elegans* Ctr protein has a 31% sequence identity with the rat protein and a 52% sequence similarity with yeast. The diagram below ***(Figure 4.4)*** shows the conservation of the putative protein between different species in the form of a phylogenetic tree.

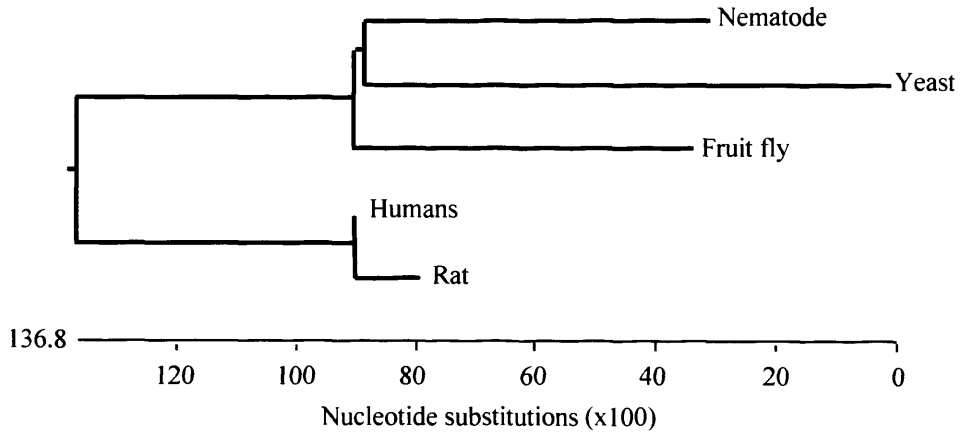


Figure 4.4 *Diagramtic representation of a phylogenetic tree of Ctr to view evolutionary relationships predicted from the multiple sequence alignment. The length of each pair of branches represents the distance between sequence pairs, while below the tree is a scale indicating the number of nucleotide substitutions. In this cladogram the unbalanced branches forces branch distances to correspond to sequence divergence (performed with DNAstar Megalign V5 using ClustalW with phylogenetic calculations based on the neighbour-joining method).*

The phylogenetic tree is based on evolution thus a highly conserved small region will infer they are more closely related in evolution though they may not align well by AA sequence. The phylogenetic tree shows that the nematode Ctr protein is most closely related to the yeast and fruit fly protein, whilst the human and rat Ctr protein are highly conserved.

4.2.2 Sequence alignment of CutC

The gene encoding the putative Cu chaperone CutC was identified in *C. elegans* by BLAST searching (using NCBI and the Wormbase database). ***Figure 4.5*** shows the AA alignment of CutC from *C. elegans*, with various species.

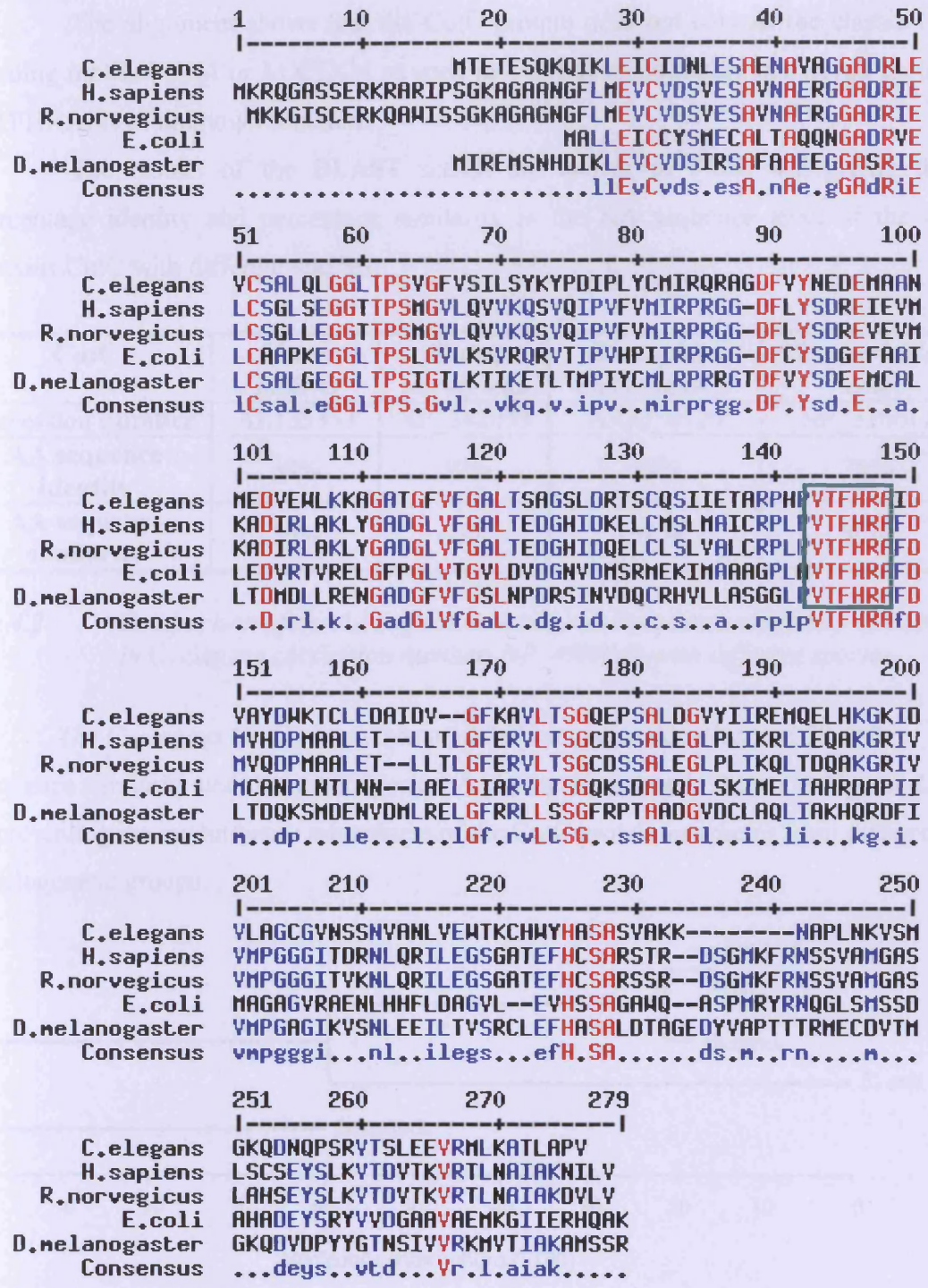


Figure 4.5 *AA sequence alignment of the putative* C. elegans *CutC with* D. melanogaster, E. coli, R. norvegicus *and* H. sapiens. *AAs in red represent a consensus of 100%, whilst those in blue represent a consensus of at least 60%. A highly conserved motif of unknown function is indicated by the green box. The alignment was performed using multiple sequence alignment with hierarchical clustering (Corpet, 1988).*

The alignment shows that the CutC protein does not contain the classic Cu binding motifs MXM or MXXXM as seen in Ctr, but has a highly conserved motif, VTFHRAFD of unknown function.

The results of the BLAST search are shown in ***Table 4.2*** giving the percentage identity and percentage similarity at the AA sequence level of the *C. elegans* CutC with different species.

CutC	*Homo sapiens*	*Rattus norvegicus*	*Drosophila melanogaster*	*Escherichia coli*
Accession number	AL133353	XP_342053	AAM50126	NP_310611
AA sequence identity	39%	39%	38%	38%
AA sequence similarity	59%	60%	58%	59%

Table 4.2 *Comparison of the AA sequence identity and sequence similarity of CutC in* C. elegans *(Accession number, NP_498855) with different species.*

The C. elegans CutC protein has a 38-39% sequence identity, and 58-60% sequence similarity with the other species. A phylogenetic tree is shown in ***Figure 4.6*** representing the evolutionary relatedness of the CutC protein in species from different phylogenetic groups.

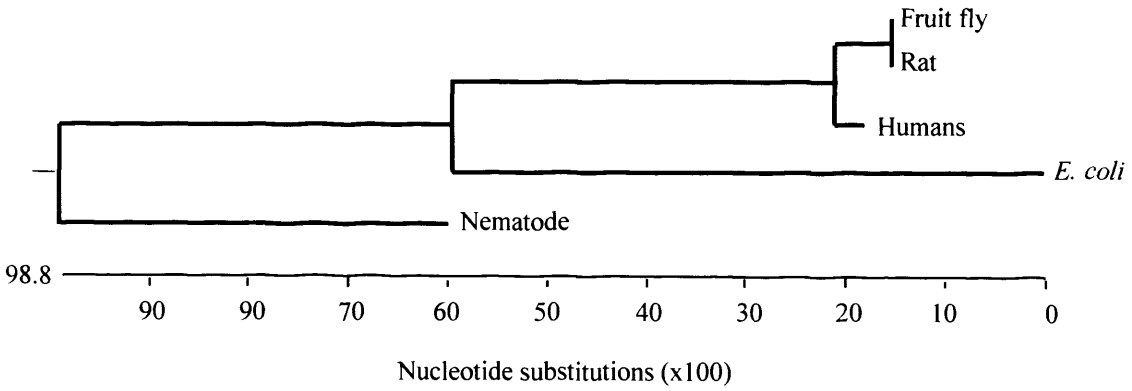


Figure 4.6 *Diagramtic representation of a phylogenetic tree of CutC to view evolutionary relationships predicted from the multiple sequence alignment. The length of each pair of branches represents the distance between sequence pairs, while below the tree is a scale indicating the number of nucleotide substitutions. In this cladogram the unbalanced branches forces branch distances to correspond to sequence divergence (performed with DNAstar Megalign V5 using ClustalW with phylogenetic calculations based on the neighbour-joining method).*

4.3 Bioinformatic analysis of Ctr and CutC

4.3.1 C. elegans *Ctr F58G6.3*

The putative *C.elegans* Ctr protein F58G6.3 is 134 AA in length, contains 3 exons and is located on chromosome IV in *C. elegans*. It forms an operon with F58G6.7 (***Figure 4.7***) which has been confirmed from electronic annotation as a Ctr protein involved in Cu transport. Its biological role has been inferred from mutant phenotypes and it is thought to be involved in gametogenesis and reproduction. It has also been shown to have a positive regulation on the body size and growth rate of *C. elegans* (WormBase web site, http://www.wormbase.org, release, date 2005). F58G6.3 and F58G6.7 have been described as two separate genes due to EST analysis, and PCR confirmation. Another Ctr family member, F58G6.9, is located about 1.2Kbp upstream of F58G6.3 (***Figure 4.7***). This Ctr is hypothetical and has not yet been confirmed as a Cu transporter.

Among individual members of the Ctr family there is little AA sequence identity; therefore bioinformatic analysis was used to infer as much information as possible about the putative *C. elegans* Ctr F58G6.3, and identify the properties characteristic of all Ctr proteins to confirm its putative role as a Cu transporter. Various bioinformatic tests were performed and it was deduced that F58G6.3 does not have an N-terminal signal peptide, a mitochondrial or nuclear localisation site nor a ER retention motif (as defined by the EXPASY bioinformatics tools (www.expasy.org)), which are characteristics found in all Ctr proteins. ***Figure 4.8*** shows the prediction of transmembrane domains in the Ctr protein based on hydrophobicity of the AA sequence.

The hydrophobicity plot predicts 3 membrane bound domains with the N-terminal on the outside and the C-terminal on the inside. PSIPRED View was used to visualise the membrane topology of the putative Ctr and the results are shown in ***Figure 4.9***.

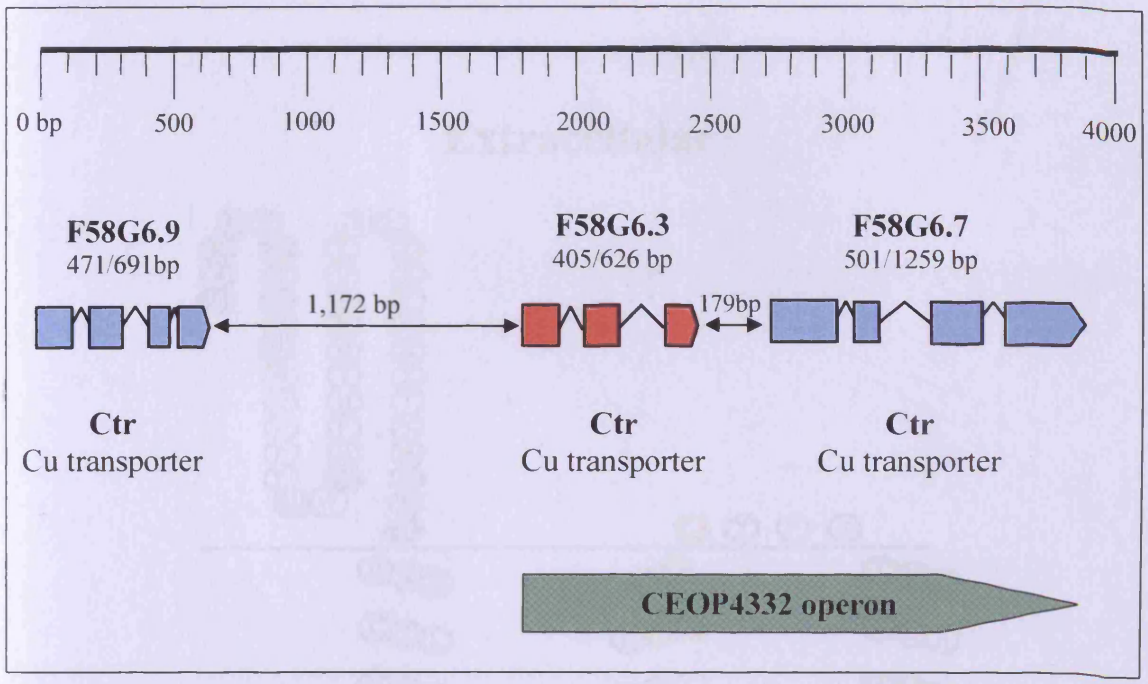


Figure 4.7 *Electronic annotation of Ctr in* C. elegans *(adapted from the WormBase web site, http://www.wormbase.org, release* WS130*).*

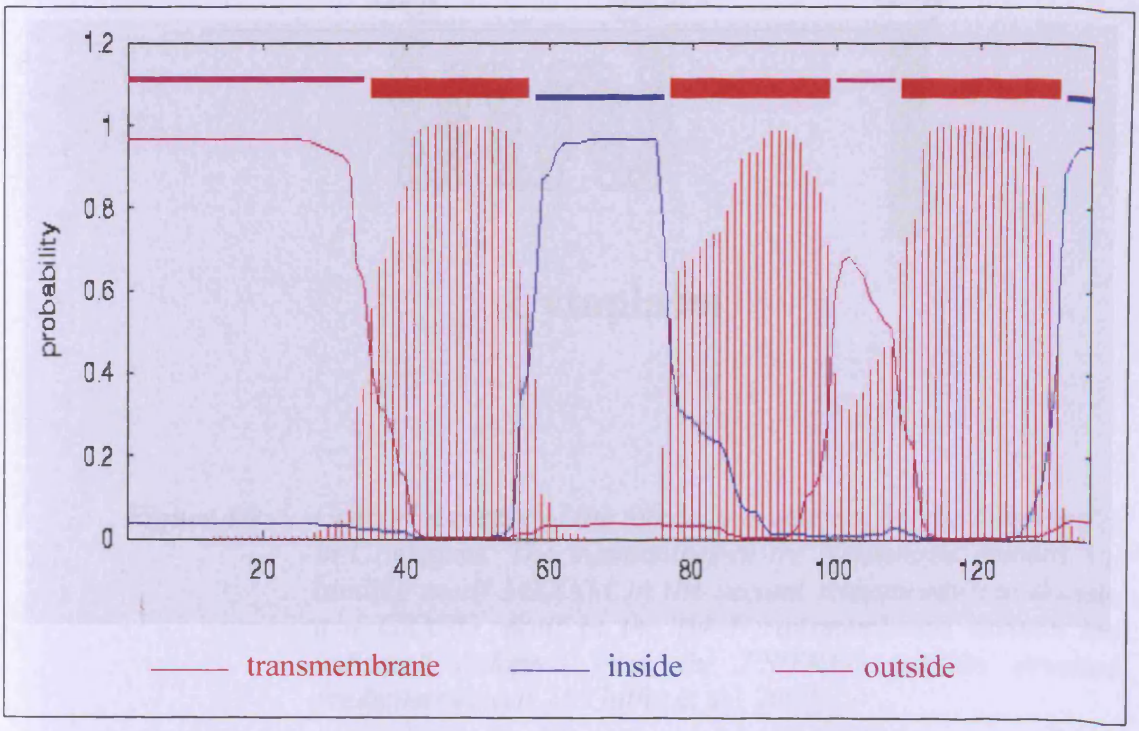


Figure 4.8 *Hydrophobicity plot predicting transmembrane domains in the putative* C. elegans *Ctr F58G6.3 (TMHMM result, Krogh* et al.*, 2001; Tusnády and Simon, 1998 and Tusnády and Simon, 2001).*

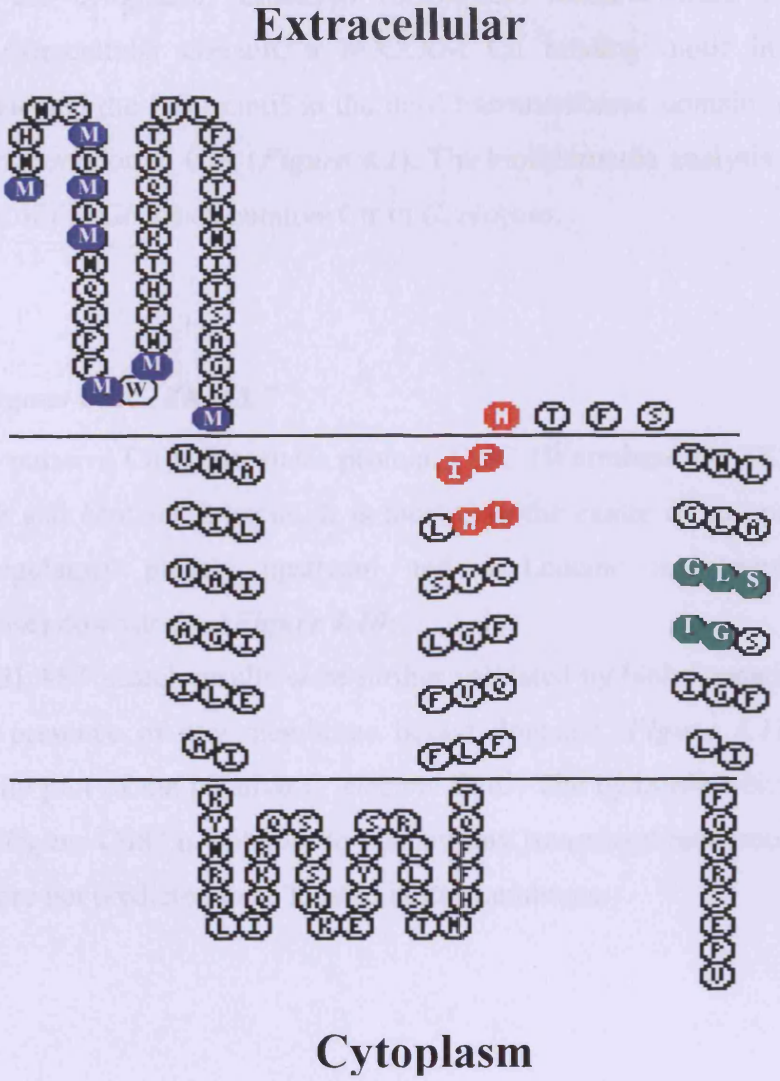


Figure 4.9 *Graphical output of the topology prediction for the putative Ctr in* C. elegans. *The methionines in the N-terminal domain, Cu binding motif MXXXM in the second transmembrane domain and GXXXG motif in the third transmembrane domain are indicated (adapted from the PSIPRED protein structure prediction server, McGuffin* et al., *2000).*

Figure 4.9 confirms that the Ctr protein F58G6.3 is likely to consist of 3 transmembrane domains with the N-terminal on the extracellular side and the C-terminal in the cytoplasm. Clustered methionine residues were found in the hydrophilic extracellular domain, a MXXXM Cu binding motif in the second transmembrane and the GG4 motif in the third transmembrane domain, which are all characteristics common to Ctrs (***Figure 4.1***). The bioinformatic analysis validates the identification of F58G6.3 as a putative Ctr in *C. elegans*.

4.3.2 C. elegans CutC, ZK353.7

The putative Cu homeostasis protein, CutC (Wormbase Id: ZK353.7 is 250 AA in length and contains 2 exons. It is located in the centre of an operon with an Ubiquitin regulatory protein upstream and a Leucine aminopeptidase (zinc metalloprotease) downstream (***Figure 4.10***).

The BLAST search results were further validated by bioinformatic analysis to identify the presence of any membrane bound domains. ***Figure 4.11*** shows the hydrophobicity plot of the putative *C. elegans* CutC. The hydrophobicity plot shows that the *C. elegans* CutC is unlikely to contain any transmembrane bound domains, and is therefore not predicted to be located in the membrane.

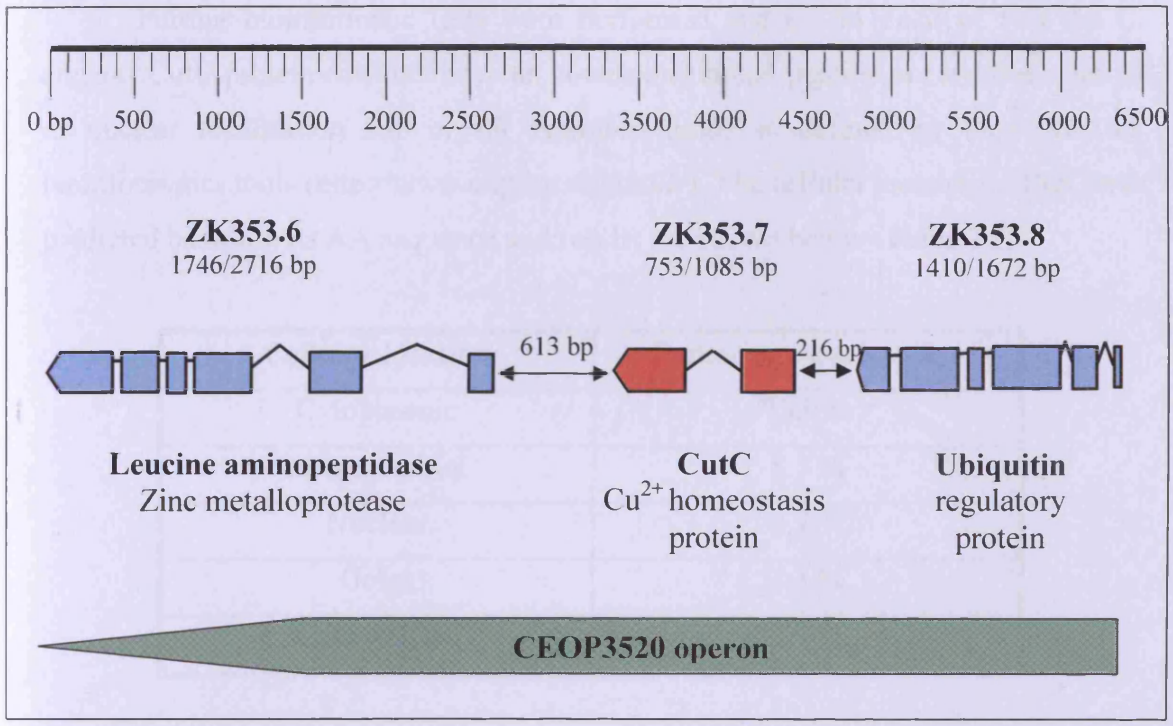


Figure 4.10 *Electronic annotation of CutC in* C. elegans *(adapted from the WormBase web site, http://www.wormbase.org, release* WS130*).*

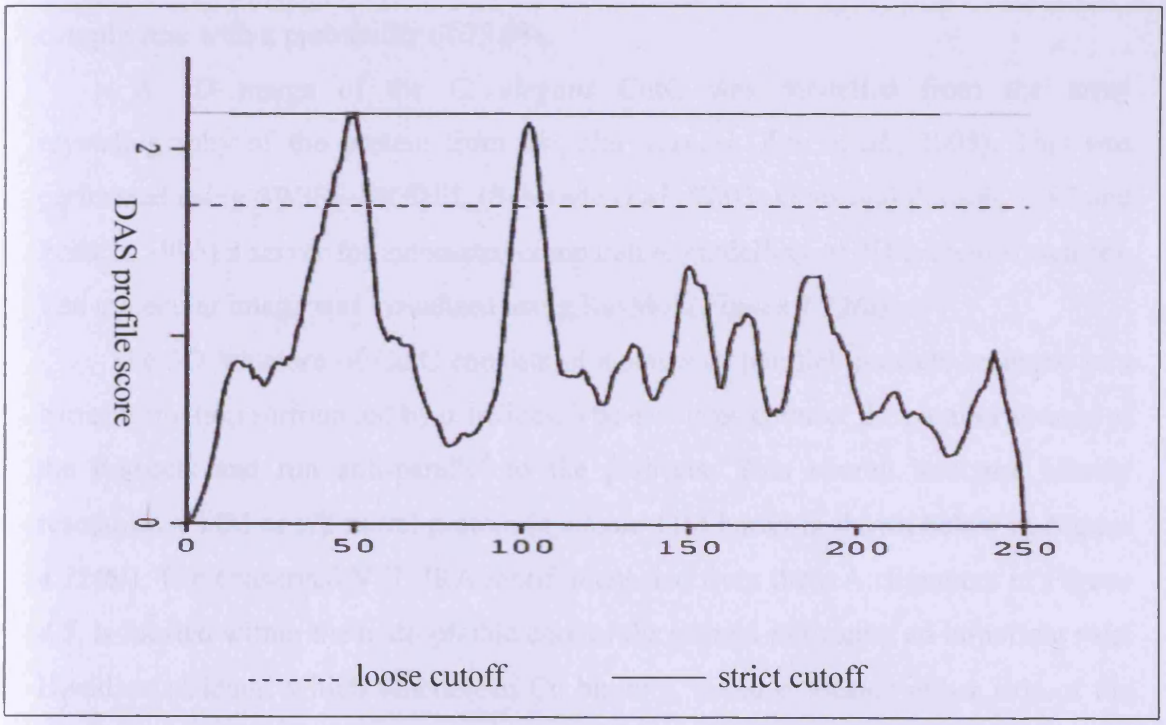


Figure 4.11 *Hydrophobicity plot of the* C. elegans *CutC protein (performed using the DAS- transmembrane prediction server; Cserzo, 1997).*

Further bioinformatic tests were performed and it was deduced that the *C. elegans* CutC protein does not have an N-terminal signal peptide nor a mitochondrial or nuclear localisation site or ER retention motif as defined by the EXPASY bioinformatics tools (http://www.expasy.org/tools/). The cellular location of CutC was predicted based on its AA sequence and results are shown below (***Table 4.3***).

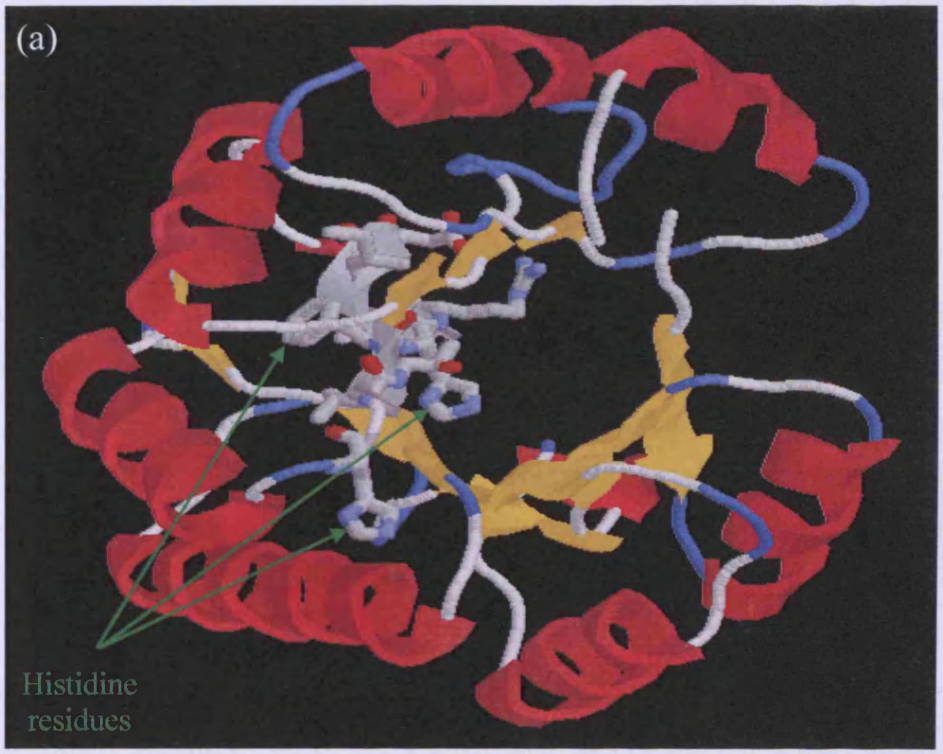
Cellular location	Percentage probability
Cytoplasmic	73.9 %
Mitochondrial	8.7 %
Nuclear	8.7 %
Golgi	4.3 %
Cytoskeletal	4.3 %

Table 4.3 Prediction of the cellular location of CutC. Performed using the *k*-NN prediction (http://www.expasy.org/tools/).

The results show that the putative *C. elegans* CutC is most likely to be cytoplasmic with a probability of 73.9%.

A 3D image of the *C. elegans* CutC was modelled from the x-ray crystallography of the protein from *Shigella flexneri* (Zhu *et al.*, 2005). This was performed using SWISS-MODEL (Schwede *et al.*, 2003; Guex and Peitsch, 1997 and Peitsch, 1995) a server for automated comparative modelling of 3D protein structures. The molecular image was visualised using RasMol (***Figure 4.12(a)***).

The 3D structure of CutC consists of a series of parallel β-sheets arranged in a barrel formation surrounded by α-helices. The α-helices connect the parallel strands of the β-sheets and run anti-parallel to the β-sheets. This overall structure closely resembles a TIM or α/β barrel protein (a classic TIM barrel is shown below in ***Figure 4.12(b)***). The conserved VTFHRA motif, identified from the AA alignment in ***Figure 4.5***, is located within the hydrophobic core of the protein indicating an important role. Histidine residues, which function in Cu binding, are also located either side of the hydrophobic core.



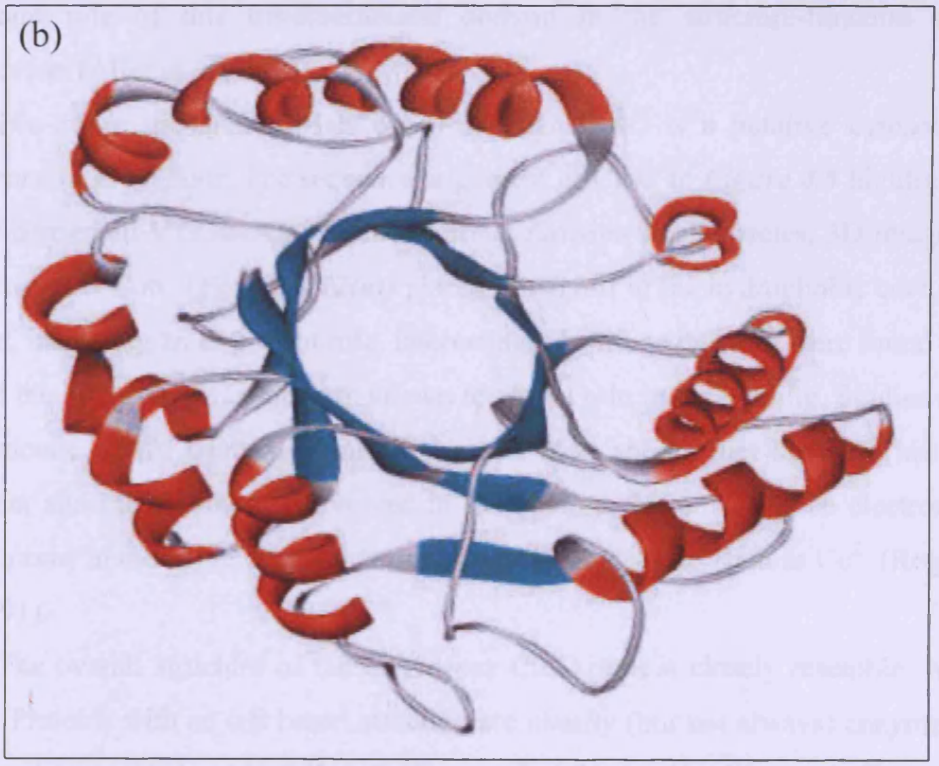


Figure 4.12 ***(a)*** *3D molecular model of the putative* C. elegans *CutC, with the AA residues from the conserved VTFHRA motif shown and the histidine residues indicated. Visualised using RasMol Biomolecular graphics for all (Sayle and Milner-White, 1995).* ***(b)*** *Topology diagram of Hevamine, an example of a TIM barrel structure (taken from* Babu, 2007 *http://www.mrc-lmb.cam.ac.uk/genomes/madanm/articles/timanal.html).*

4.4 Discussion

Bioinformatic analysis confirmed that Ctr is a putative membrane-bound Cu transporter in *C. elegans*. The three-transmembrane protein has characteristics found in all Ctr family members. The predicted protein topology in ***Figure 4.9*** contains all the conserved features common to all Ctr proteins, as shown in ***Figure 4.1***. The *C. elegans* Ctr has a cluster of methionine residues in the N-terminal extracellular domain which have been shown to have a role in Cu binding (Puig *et al.*, 2002) and also contains the Cu binding motif, MXXXM. Recent studies have revealed that the MXXXM motif in the second transmembrane domain plays a vital role in the chemistry of Cu sensing and uptake (Aller *et al.*, 2004). The Ctr protein has the highly conserved GG4 motif in the third transmembrane domain which has recently been found to be essential for Ctr oligomerisation and stabilisation of transmembrane helix-helix interactions. The presence of an invariantly conserved GG4 motif in the third transmembrane domain of all of the known members of the Ctr family suggests an important role of this transmembrane domain in the structure-function of Ctr transporters (Aller *et al.*, 2004).

Bio-informatic analysis has confirmed that CutC is a putative cytosolic Cu chaperone in *C. elegans*. The sequence alignment of CutC in ***Figure 4.5*** highlighted a highly conserved VTFHRA motif of unknown function in all species. 3D imagery of the *C. elegans* CutC (***Figure 4.12(a)***) placed this motif in the hydrophobic core of the protein, indicating an important role. Interestingly histidine residues were found either side of the protein pore, which are known to play a role in Cu binding. Studies of the 3D structure of Cu containing metalloproteins have shown that the AAs histidine, cysteine, and methionine are involved in Cu binding. These AA have electron pair donor atoms in their side chains that can bond with metal ions such as Cu^{2+} (Rogers *et al.*, 1991).

The overall structure of the *C. elegans* CutC protein closely resembles an α/β barrel. Proteins with an α/β barrel structure are usually (but not always) enzymes and are found in approximately 10% of all enzymes with known structures. Despite the structural homology, the members of this large family of proteins catalyze a very wide range of different reactions and can also have nonenzymatic roles in the cell including binding and transport proteins (Reardon and Farber, 1995 and Nagano *et al.*, 1999).

Figure 4.10 shows that CutC is located in the centre of an operon with an ubiquitin regulatory protein upstream and a leucine aminopeptidase downstream. *C. elegans* and its relatives are unique among animals in having operons. These operons are regulated multigene transcription units, in which polycistronic pre-messenger RNA (pre-mRNA coding for multiple peptides) is processed to monocistronic mRNAs (Blumenthal *et al.*, 2002). Operons are not random gene assemblages and usually co-regulate genes that make proteins with related functions (Blumenthal. and Gleason, 2003).

The upstream ubiquitin regulatory protein UBXD2, contains a UBX domain which is found in ubiquitin-regulatory proteins. The attachment of ubiquitin to proteins by ubiquitination causes inactivatation and acts as a tag by which a protein-transport machinery ferries the protein to the proteasome for degradation. The ubiquitination pathway degrades a multitude of cellular proteins, including cell cycle regulators, growth- and differentiation-controlling factors, transcriptional activators, cell-surface receptors and ion channels, endoplasmic reticulum proteins, antigenic proteins and abnormal and misfolded proteins (Hall. *et al.*, 2005). The proteins targeted by this system are short-lived proteins, many of which are regulatory proteins, whose actions are controlled in part by rapid synthesis and degradation.

Ubiquitination can also be used to modify a protein's activity, to change its subcellular location, or to alter protein-protein interactions. Different types of ubiquitin modifications are capable of transmitting unique signals. The addition of a single ubiquitin molecule (mono-ubiquitination) is a reversible modification used to alter the function and localisation of key proteins in several processes, including membrane trafficking, transcription and histone function.

The gene downstream of CutC, lap-1, encodes a homolog of the zinc metalloprotease aminopeptidase. Leucine aminopeptidases are exopeptidases which are presumably involved in the processing and regular turnover of intracellular proteins; however, their precise function in cellular metabolism remains to be established (Bartling and Weiler, 1992). Leucine aminopeptidases cleave leucine residues preferentially from the N-terminal of polypeptide chains and are implicated in the final steps of intracellular proteolysis (Saric *et al.*, 2004).

It has been suggested that the final steps in ubiquitin-proteasome pathway involves aminopeptidases, which very rapidly digest the small proteasome products

(Saric *et al.*, 2004). Therefore the ubiquitin regulatory protein and leucine aminopeptidase found either side of CutC in the operon are likely to be co-expressed as it is probable that they work together in protein regulation.

Chapter 5

Expression studies of Ctr and CutC in *C. elegans*

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5.1 Introduction to Quantitative PCR

Quantitative PCR was used to assess the gene expression level of Ctr and CutC in *C. elegans*, to determine changes in the relative expression over different $CuSO_4$ concentrations. The technique uses florescence monitoring of the DNA products amplified from cDNA samples by PCR-thermal cycling. At the end of each extension step the products formed were monitored by measuring the level of fluorescence and taking into account the level of expression of a control gene the relative expression level was determined. In this study the housekeeping gene rpa-1 was used as the control gene. This is a ribosomal gene which encodes for the 60s acidic ribosomal subunit protein and is expressed at a stable basal level (Swain *et al.*, 2004).

5.2 *C. elegans* exposure and sample preparation

Nematodes were cultured in liquid media and exposed to 0, 100, 500 and 2000μM $CuSO_4$ over a 24 hour period as described in ***Section 2.4***. RNA was extracted from the nematodes using Tri-reagent (***Section 2.8.2)***. The RNA was visualised by agarose gel electrophoresis (***Figure 5.1***).

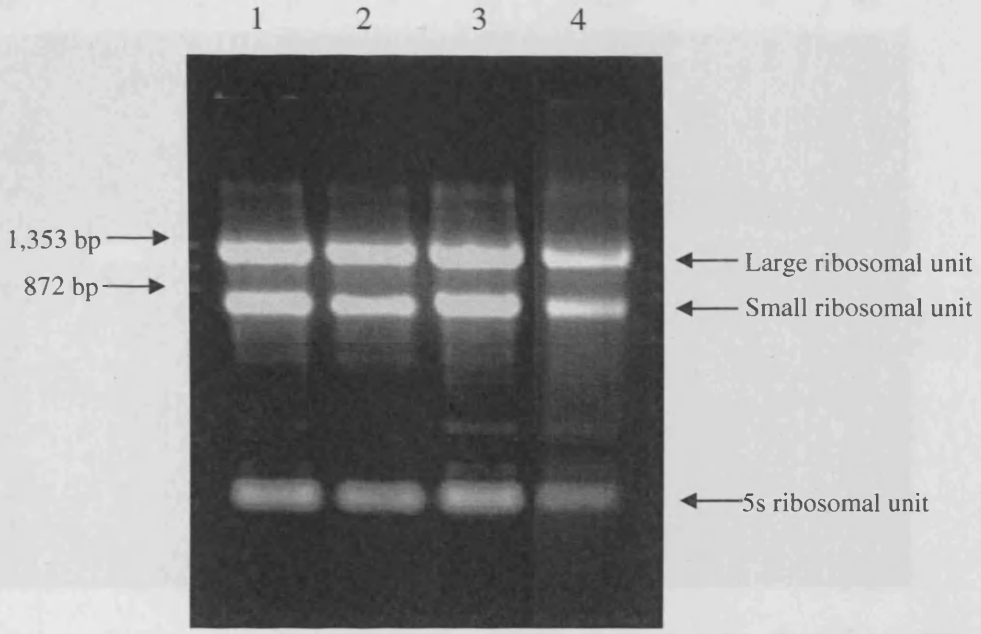

Figure 5.1 *Agarose gel electrophoresis of* C. elegans *total RNA. Lane 1:* C. elegans *exposed to 0μM $CuSO_4$, lane 2: 100μM $CuSO_4$, lane 3: 500μM $CuSO_4$ and lane 4: 2000μM $CuSO_4$.*

The RNA was quantified, if necessary concentrated by ethanol precipitation (***Section 2.8.6***) and 2μg was used to generate cDNA in a RT reaction (***Section 2.10.3***). Each cDNA sample was diluted 1:10 with HPLC H_2O prior to QPCR and analysed in triplicate.

5.3 Preparation of standards

Gene specific primers were used to amplify Ctr, CutC and rpa-1 from *C. elegans* cDNA (***Section 2.10.3***). The PCR product was visualised by agarose gel electrophoresis (***Figure 5.2***), and gel extracted (***Section 2.9.2***).

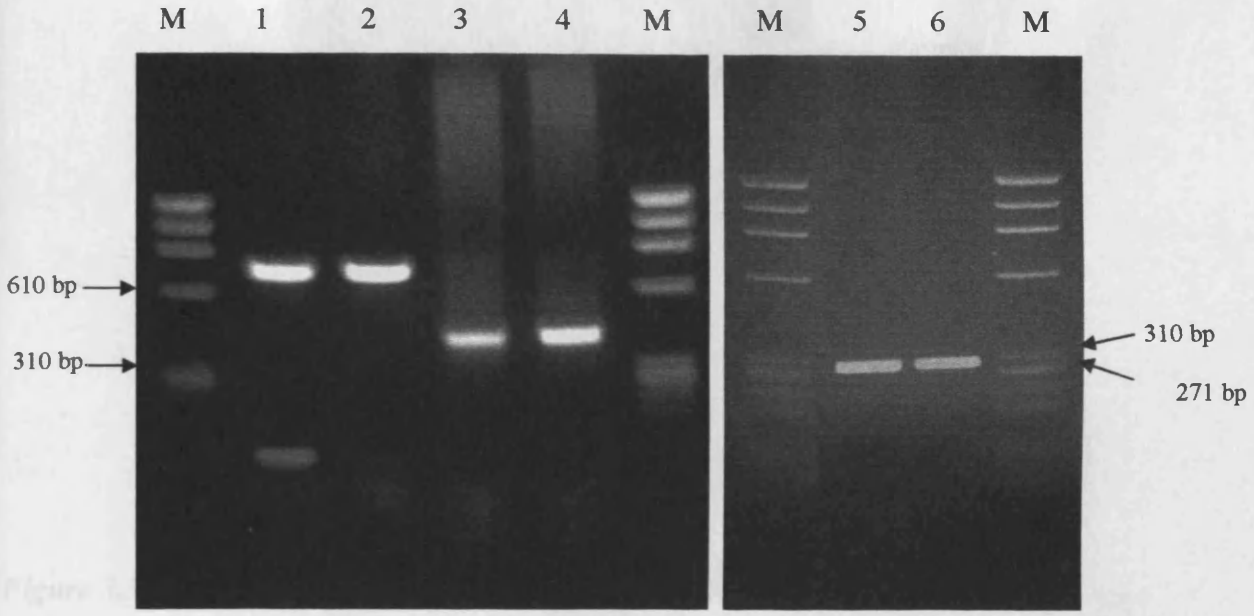

Figure 5.2 *Agarose gel electrophoresis of PCR products from the RT reaction. Lane M: øX174H3HaeIII digest marker, lane 1: CutC PCR (TM 58°C), lane 2: CutC PCR (TM 62°C), lane 3: Ctr PCR (TM 48°C), lane 4: Ctr PCR (TM 50°C), lane 5: rpa-1 PCR (TM 58°C) and lane 6: rpa-1 PCR (TM 62°C).*

The extracted PCR product was cloned into pGEM-T using the TA cloning system (***Section 2.13.3***) and the plasmid DNA was purified using the Wizard Plus SV Miniprep (***Section 2.13.1***). Positive clones were selected by PCR (***Section 2.13.6***) using primers designed following the guidelines in ***Section 2.11.2***. In ***Figure 5.3*** the agarose gel electrophoresis shows the PCR screen for positive clones using the gene specific QPCR primers. The inserts of the plasmids were then sequenced using universal M13 primers to confirm the identity of the insert ***(Section 2.14)***. A standard dilution series of calibration standards were prepared by diluting each purified plasmid to a known concentration, in the range of 1ng/µl to 100ag/µl.

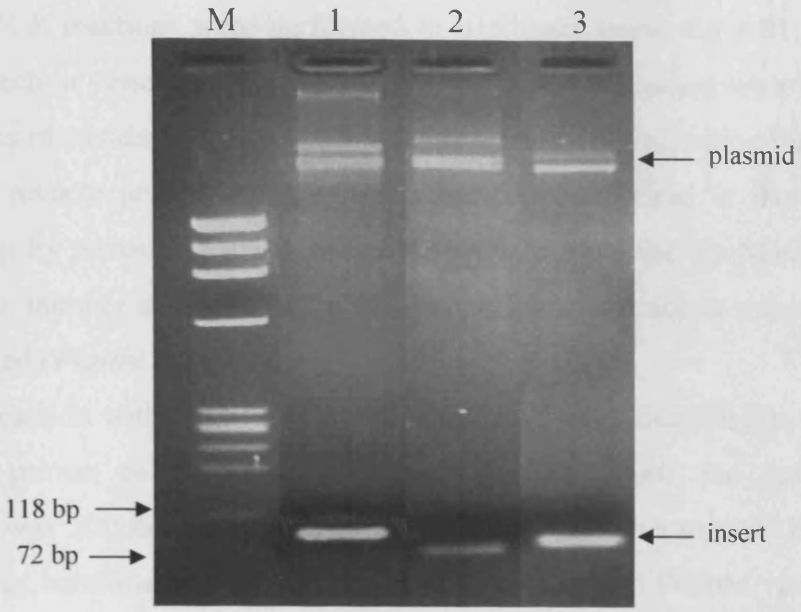

Figure 5.3 *Agarose gel electrophoresis of the PCR screen for positive clones from plasmid DNA. Lane M: øX174H3HaeIII digest marker, lane 1: Ctr, lane 2: CutC and lane 3: rpa-1.*

5.4 Optimisation of primers

The Ctr, CutC and *rpa-1* primers were optimised prior to TaqMan® probe PCR quantification using 250pg plasmid DNA as a template (***Section 2.11.5***). Three different primer concentrations were tested resulting in 9 primer combinations (***Table 5.1***).

Final primer conc. (nM)	Primer type	Final primer conc. (nM)	Primer type	Final primer conc. (nM)	Primer type
50	Forward	50	Forward	50	Forward
50	Reverse	300	Reverse	900	Reverse
300	Forward	300	Forward	300	Forward
50	Reverse	300	Reverse	900	Reverse
900	Forward	900	Forward	900	Forward
50	Reverse	300	Reverse	900	Reverse

Table 5.1 *Combination of primer concentrations used in the QPCR optimisation.*

The PCR reactions were performed in triplicates using the ABI Prism® 7700 sequence detection system. Negative controls were analysed along with two different concentrations of standards for comparison (1ng/µl and 10fg/µl) using 900nM of both forward and reverse primer. Product formation was monitored at the end of each extension step by measuring the fluorescence emitted from the TaqMan® probes and Ct values (the number of cycles before the probe fluoresces above a threshold level) were calculated (***Figure 5.4***).

The reaction with the lowest Ct value and best amplification rate was taken as the optimal primer concentration. For both Ctr and CutC the optimal primer combination was 300nM forward primer and 900nM reverse primer. For rpa-1 the optimal primer combination was 50nM forward primer and 900nM reverse primer.

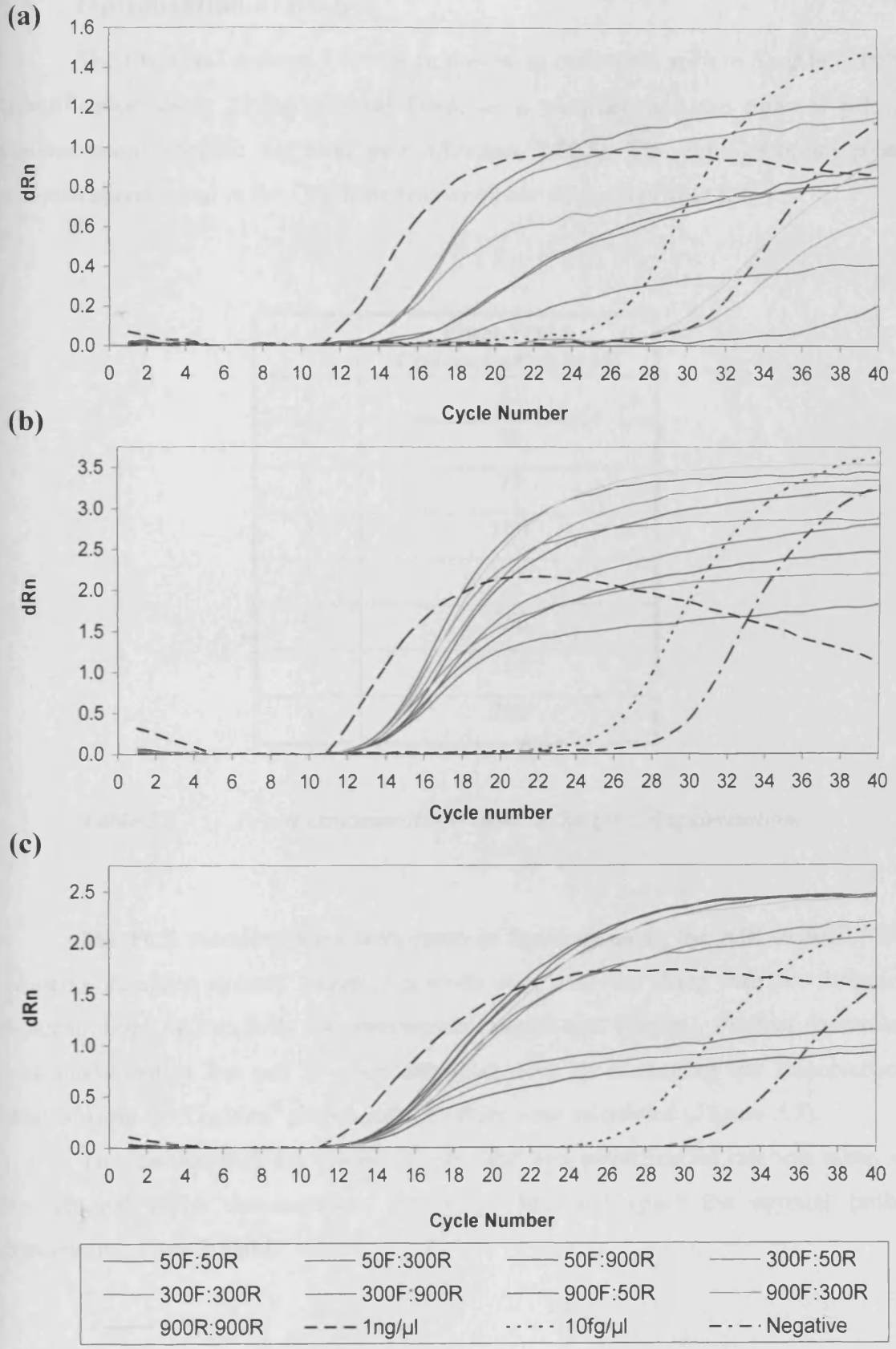

Figure 5.4 *Primer optimisation for (a) Ctr, (b) CutC and (c) rpa-1 QPCR.*

5.5 Optimisation of probes

The Ctr, CutC and rpa-1 QPCR probes were optimised prior to TaqMan® PCR quantification using 250pg plasmid DNA as a template and the optimal primer concentrations specific for each gene (***Section 2.11.5***). The eight different probe concentrations tested in the QPCR optimisation are shown in ***Table 5.2***.

	Final Probe Concentration (nM)
1	25
2	50
3	75
4	100
5	125
6	150
7	175
8	200

Table 5.2 *Probe concentrations used in the QPCR optimisation.*

The PCR reactions were performed in triplicate using the ABI Prism® 7700 sequence detection system. Negative controls were analysed along with two different concentrations of standards for comparison (1ng/µl and 10fg/µl). Product formation was monitored at the end of each extension step by measuring the fluorescence emitted from the TaqMan® probes and Ct values were calculated (***Figure 5.5***).

The reaction with the lowest Ct value and best amplification rate was taken as the optimal probe concentration. For Ctr, CutC and rpa-1 the optimal probe concentration was 200nM.

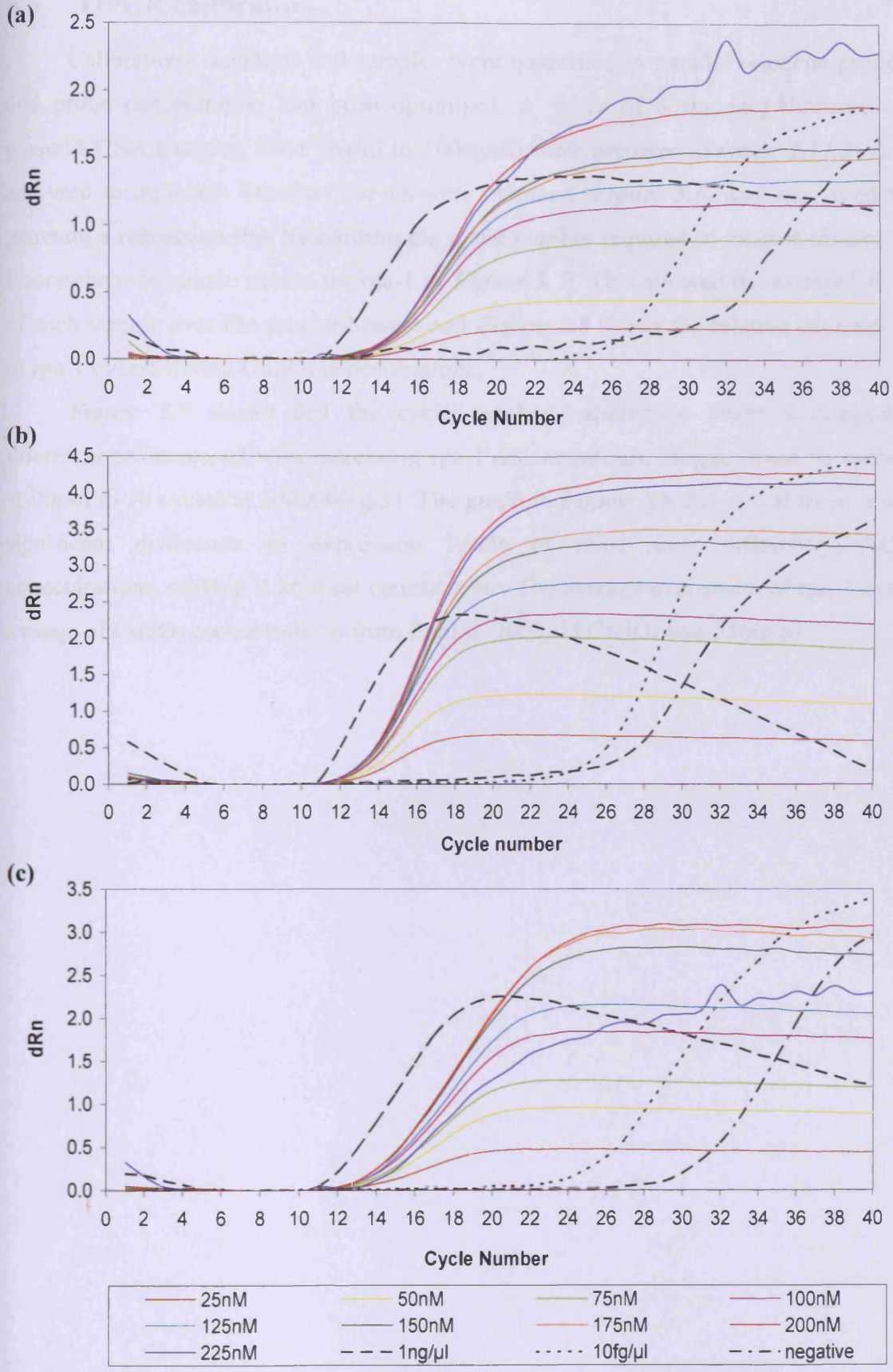

Figure 5.5 *Probe optimisation for (a) Ctr, (b) CutC and (c) rpa-1 QPCR.*

5.6 QPCR calibration

Calibrations standards and samples were quantified in parallel once the primer and probe concentration had been optimised. A series of 8 standard dilutions of plasmid DNA (ranging from 1ng/µl to 100ag/µl) were prepared (***Section 2.11.3***) and analysed in triplicate. Standard curves were obtained (***Figure 5.6***) and were used to generate a regression line by plotting the cycle number required to attain a threshold fluorescence (example shown for rpa-1 in ***Figure 5.7***). This allowed the extrapolation of each sample over the standard range and ***Figure 5.8*** shows the relative expression of rpa-1 over different $CuSO_4$ concentrations.

Figure 5.7 shows that the cycle number required to attain a threshold fluorescence decreased with increasing rpa-1 concentrations, ranging from 30 cycles at 0fg/µl to 10 cycles at 1000000fg/µl. The graph in ***Figure 5.8*** shows that there is no significant difference in expression levels of rpa-1 over different $CuSO_4$ concentrations, making it an ideal control gene. The average expression of rpa-1 over a range of $CuSO_4$ concentrations from 0µM to 2000µM $CuSO_4$ was 556fg/µl.

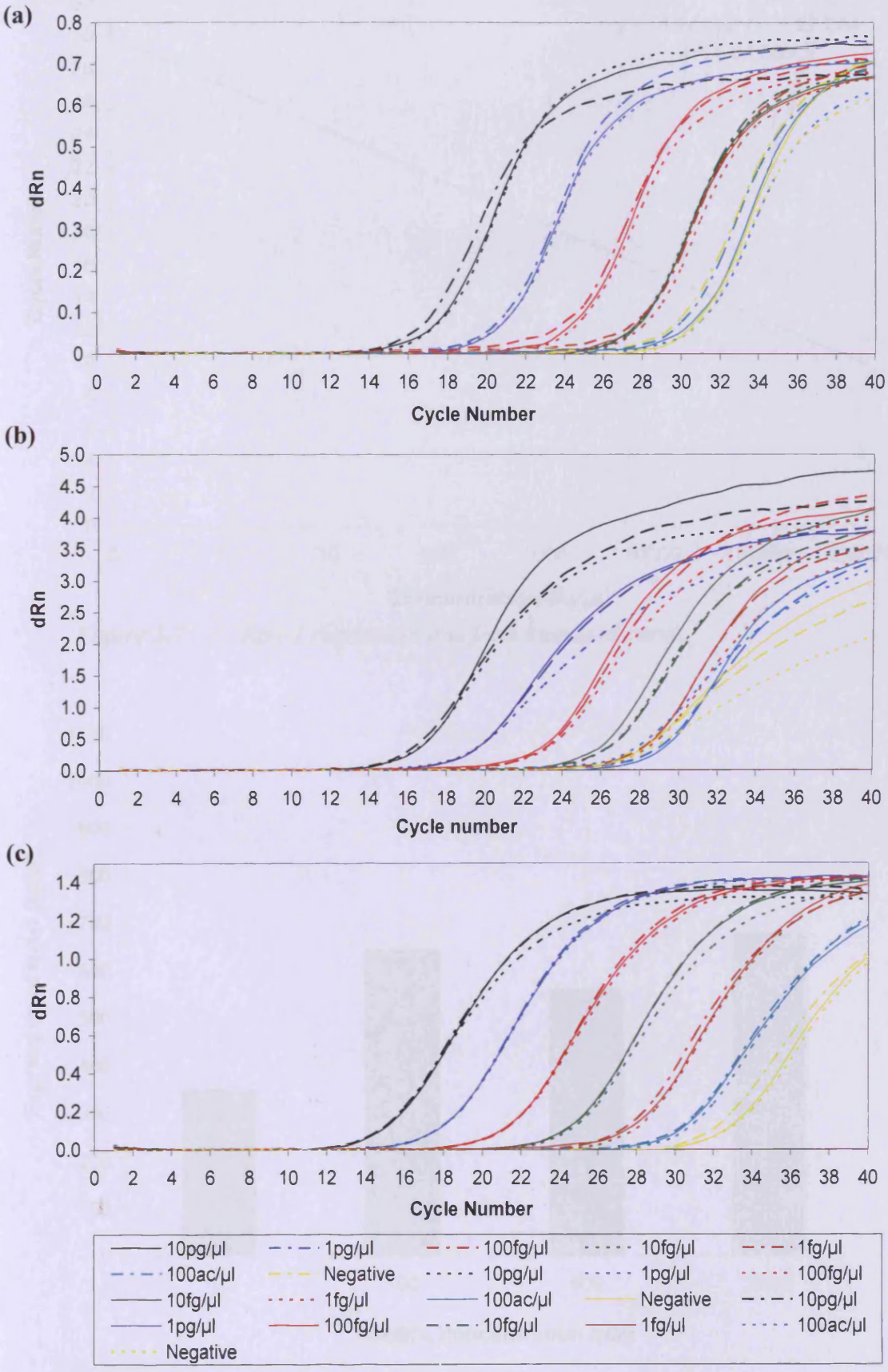

Figure 5.6 *Standard curves for (a) Ctr, (b) CutC and (c) rpa-1 QPCR.*

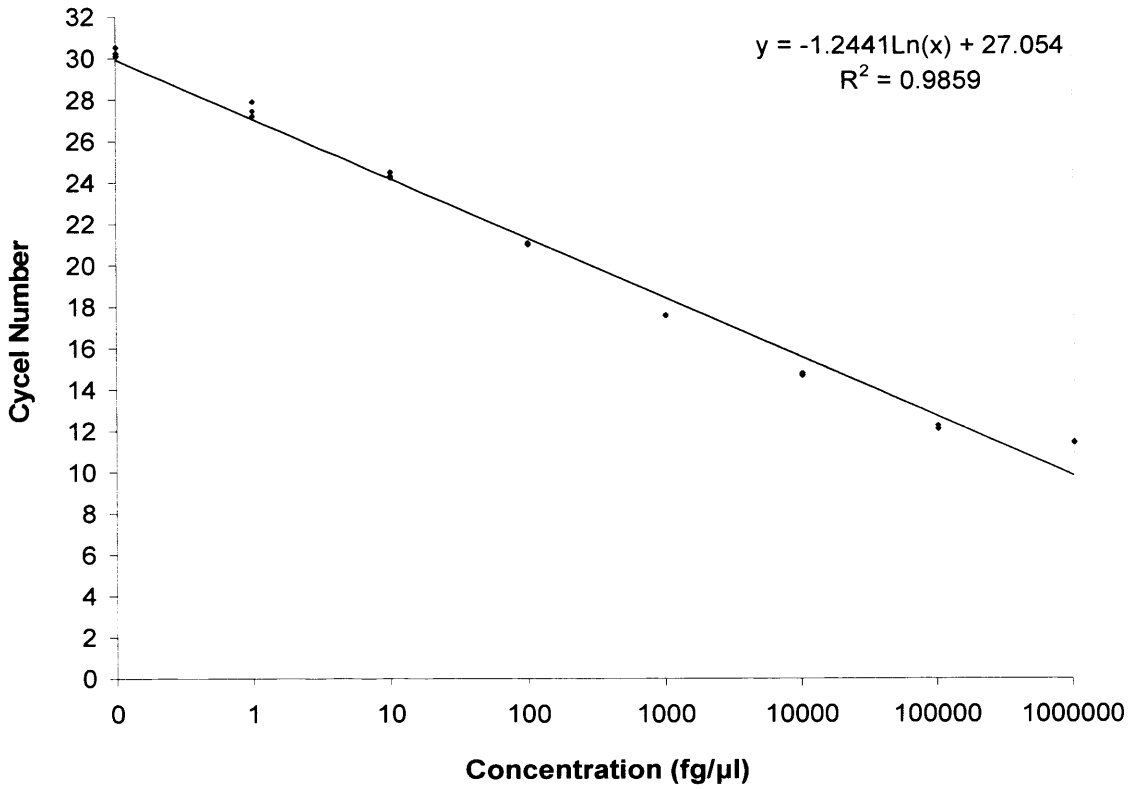

Figure 5.7 *Rpa-1 regression line from standard curve.*

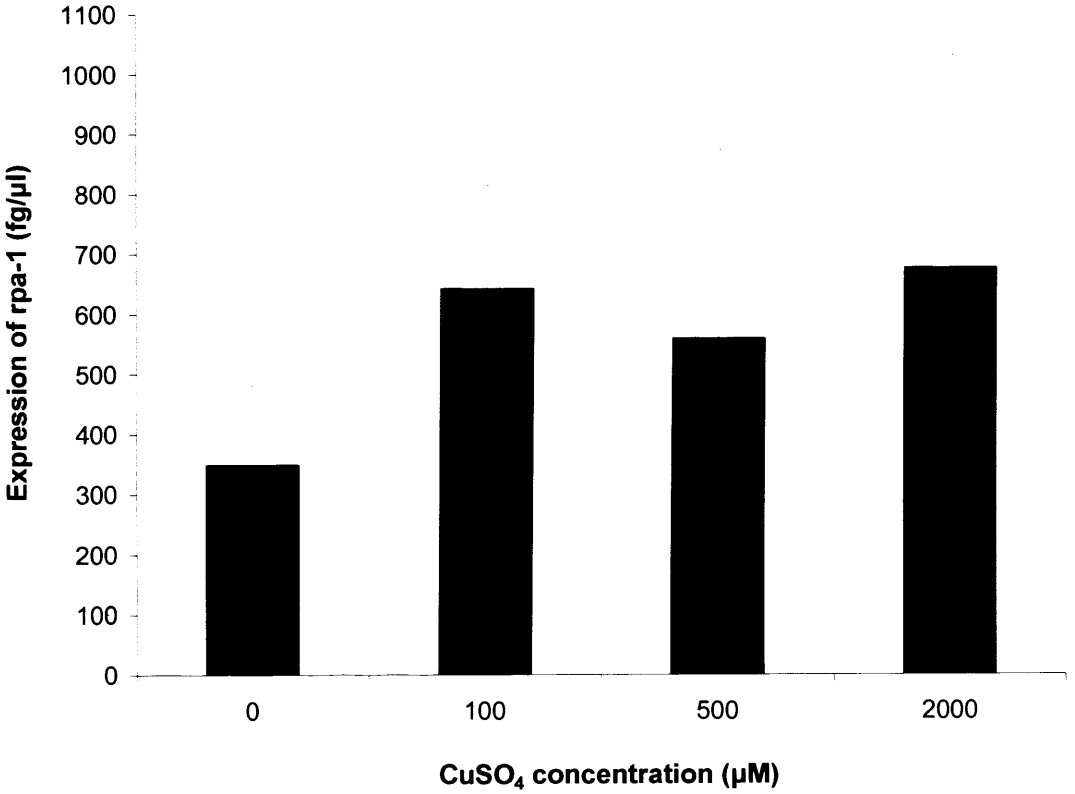

Figure 5.8 *Relative expression of rpa-1 over a range of $CuSO_4$ concentrations, ± SEM (n=5).*

5.7 Expression of Ctr and CutC in wild-type *C. elegans*

The relative expression of Ctr and CutC over a range of $CuSO_4$ concentrations was determined by TaqMan® probe technology on the ABI Prism® 7700 Sequence Detection System as described in ***Section 2.11.6***, using the optimal primer and probe conditions determined for the gene. Five biological replicates were prepared for each test sample, and for each biological replicate the cDNA samples were analysed in triplicate. Expression levels were normalised against the control gene rpa-1 and the results are shown in ***Figure 5.9***.

The results in ***Figure 5.9*** show that the relative expression of both Ctr and CutC in *C. elegans* decreases over increasing $CuSO_4$ concentrations. With Ctr the expression levels were significantly less at 100μM $CuSO_4$ ($p \leq 0.02$), 500μM $CuSO_4$ ($p \leq 0.00$) and 2000μM $CuSO_4$ ($p \leq 0.00$) than at 0μM $CuSO_4$. The relative expression of CutC in *C. elegans* $CuSO_4$ was significantly greater at 0μM compared to 500μM and 2000μM $CuSO_4$ ($p \leq 0.012$ and $p \leq 0.006$ respectively). At 2000μM $CuSO_4$ the relative expression level of CutC was also significantly less than at 100μM $CuSO_4$ ($p \leq 0.002$).

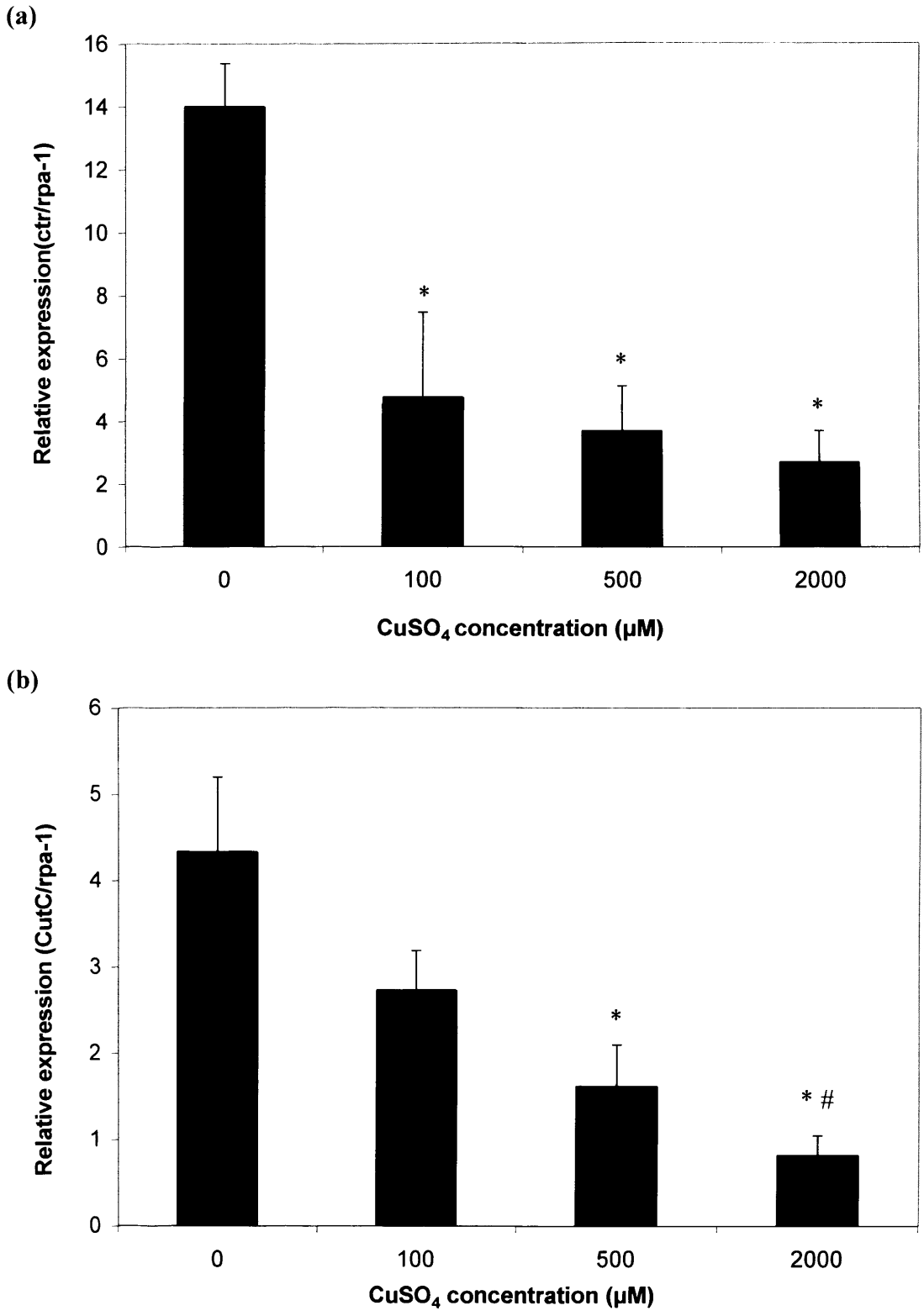

Figure 5.9 *Relative gene expression over different $CuSO_4$ concentrations for (a) Ctr and (b) CutC, ± SEM (n=5). * denotes a significant difference from 0µM $CuSO_4$ and # denotes a significant difference from 100µM $CuSO_4$.*

5.8 Expression of Ctr and CutC in *C. elegans* exposed to RNAi

5.8.1 Introduction to RNAi

RNA-mediated interference (RNAi) describes the use of exogenous RNA to interfere with the function of an endogenous gene (***Figure 5.10***). The process of RNAi was first discovered in *C. elegans* as a response to dsRNA, which resulted in a sequence specific silencing (Hannon, 2002). The target gene is inactivated by a specific break down of the mRNA, making it an ideal method for rapid identification of *in vivo* gene function (Simmer *et al.*, 2003). RNAi and related phenomena are natural evolutionary conserved mechanisms, which are thought to protect organisms from invasion by both exogenous (e.g. viruses) and endogenous (e.g. mobile genetic elements) genetic parasites (Bernstein *et al.*, 2001).

Current evidence indicates a model in which RNAi blocks a post-transcriptional step in gene expression (as promoter and intron sequences are entirely ineffective in RNAi) and suggests possible similarities with post-transcriptional gene silencing (PTGS) in plants (Grishok, *et al.*, 2000).

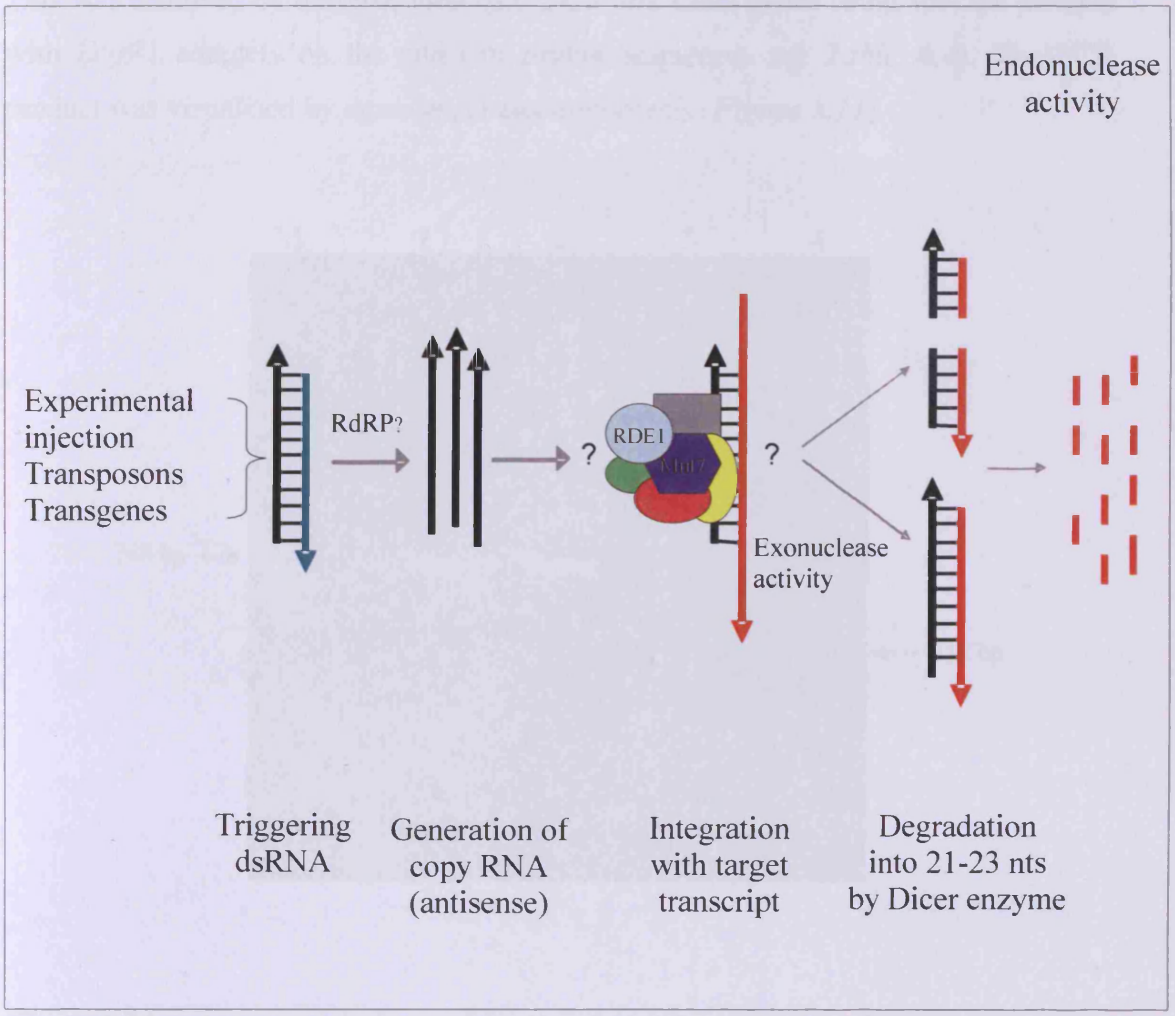

Figure 5.10 *Proposed mechanism of RNAi: dsRNA is triggered in the organism and an RNA-dependant RNA polymerase (RdRP) amplifies the antisense RNA. The anitisense RNA hybridises to the endogenous target RNA and a multi- protein complex is formed, of which two are known; RDE1 and Mut7. The endogenous mRNA is degraded into 21-23 nucleotides by the enzyme Dicer (adapted from Bosher and Labouesse, 2000).*

5.8.2 Preparation of target genes

C. elegans RNA was used to synthesise cDNA in a RT-PCR (***Section 2.10.3***). This was followed by amplification of the Ctr and CutC genes using specific primers with *Eco*R1 adapters on the end (for primer sequences see ***Table A.4***). The PCR product was visualised by agarose gel electrophoresis (***Figure 5.11***).

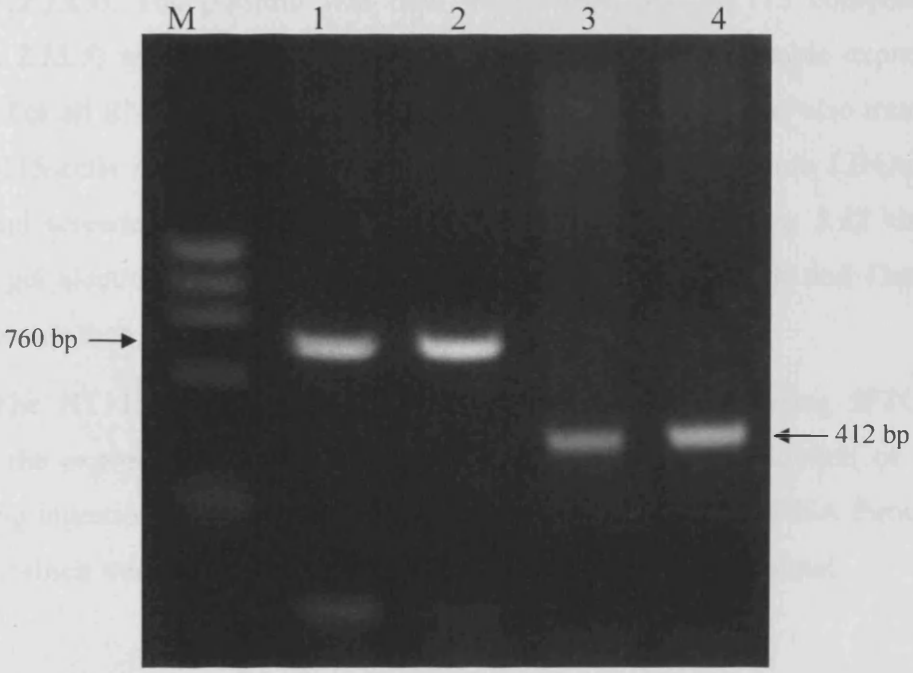

Figure 5.11 *Agarose gel electrophoresis of optimisation of the PCR amplification. Lane M: øX174H3HaeIII digest marker, lane 1: CutC (annealing temperature at 54°C), lane 2: CutC (annealing temperature at 56°C), lane 3: Ctr (annealing temperature at 48°C) and lane 4: Ctr (annealing temperature at 50°C).*

The PCR product was purified by gel extraction (***Section 2.9.2***), ligated into the pGEM-T vector using the TA cloning system and transformed into DH5α competent cells (***Section 2.13***). The plasmid was purified (***Section 2.13.1***) and positive clones were identified by restriction digest with *Eco*RI (***Section 2.13.6***). The digested DNA was purified by gel extraction and the *Eco*R1 overhangs created sticky ends for sub-cloning into the RNAi vector ppD129.36 (***Section 2.13.3***).

5.8.3 Preparation of the RNAi consruct

The RNAi vector ppD129.36 was digested with the restriction enzyme *Eco*R1 to create sticky ends (***Section 2.12.1***) and the digests were visualised on an agarose gel (***Section 2.9.1***). The linearised vector was excised from the gel and purified (***Section 2.9.2***). An appropriate amount of the target gene was ligated into ppD129.36 vector containing T7 promoters flanking each side of the multiple cloning site (***Section 2.13.3***). The plasmid was then transformed into HT115 competent cells (***Section 2.13.5***) which are deficient in RNase III, allowing the stable expression of dsRNA. For all RNAi experiments the empty ppD129.36 vector was also transformed into HT115 cells and used as a control. The cells were seeded onto LB+Amp agar plates and screened by restriction digest (***Section 2.13.6***). ***Figure 5.12*** shows the agarose gel electrophoresis of the restriction digest screen for Ctr and CutC. The construct was then confirmed by sequencing (***Section 2.14***).

The HT115 bacteria were grown on NGM agar containing IPTG which induces the expression of the T7 polymerase allowing the production of dsRNA. Following ingestion of the bacteria the *C. elegans* absorbed the dsRNA through their intestine which was then subsequently distributed throughout the animal.

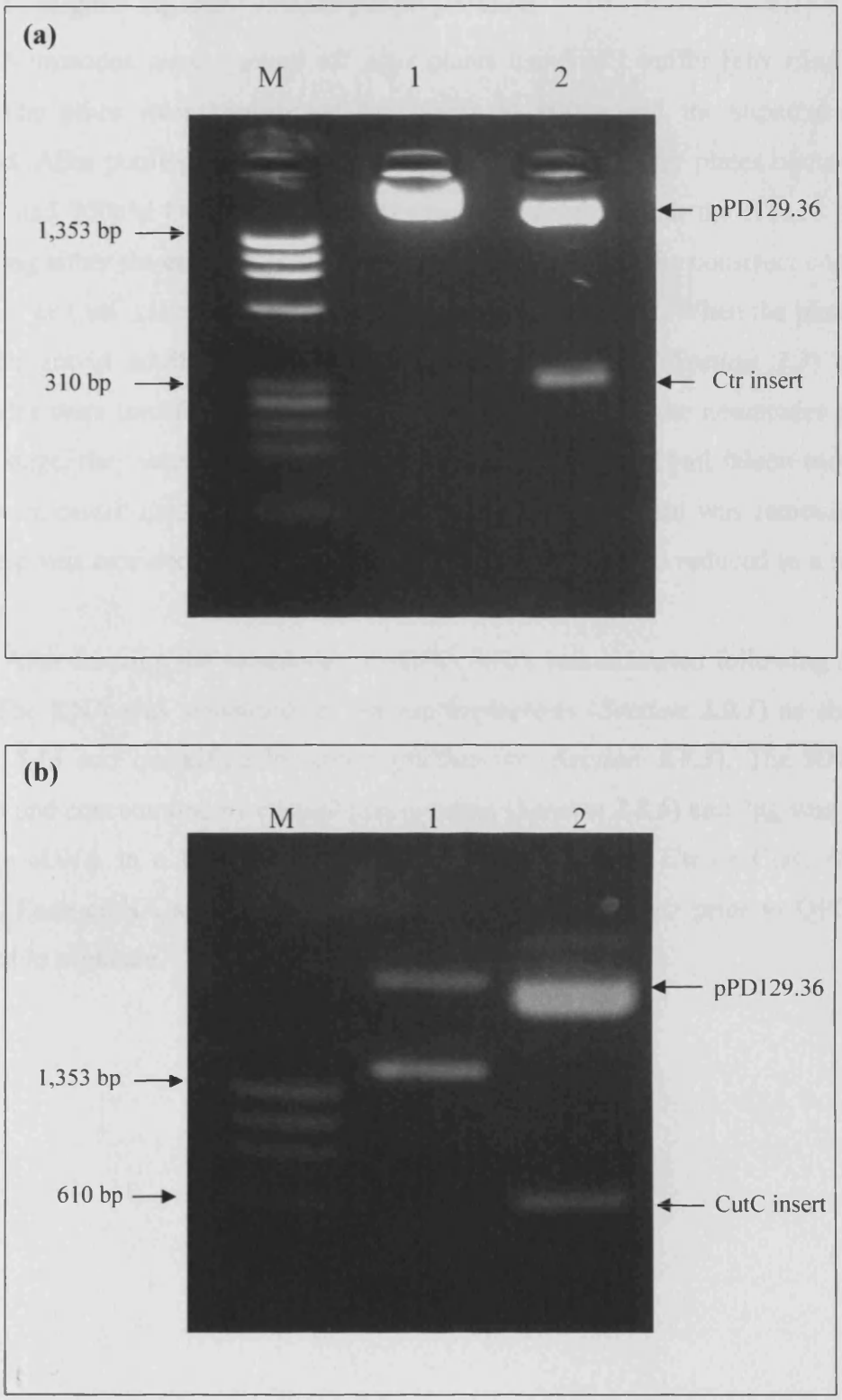

Figure 5.12 *Agarose gel electrophoresis of the restriction digest screen for (a) Ctr and (b) CutC. Lane M: øX174H3HaeIII digest marker, lane 1: pPD129.36 plasmid preparation, lane 2: pPD129.36 digest with* Eco*R1, showing the excised insert.*

5.8.4 C. elegans exposure and sample preparation

Nematodes were washed off agar plates using M9 buffer into 15ml falcon tubes. The tubes were centrifuged for 2 min at 2000g and the supernatant was removed. After pooling the nematodes were aliquoted onto agar plates containing 0, 20, 100 and 500μM $CuSO_4$. The agar plates were streaked with the HT115 bacteria containing either the empty RNAi vector as a control, or with the construct containing either Ctr or CutC cloned into the pDD129.36 vector for RNAi. When the plates were full with gravid adults an egg preparation was performed (***Section 2.3***) and the nematodes were transferred back onto the agar plates. When the nematodes reached the L4 stage, they were washed off the plates using M9 into 15ml falcon tubes. The tubes were centrifuged for 2 min at 2000g and the supernatant was removed. This wash step was repeated with M9 (5ml) and the supernatant was reduced to a minimal volume.

After freezing the nematodes at -80°C, RNA was extracted following ***Section 2.8.2***. The RNA was visualised by gel electrophoresis (***Section 2.9.1***) as shown in ***Figure 5.13*** and quantified by spectrophotometry (***Section 2.9.3***). The RNA was purified and concentrated by ethanol precipitation (***Section 2.8.6***) and 2μg was used to generate cDNA in a RT reaction using specific primers to Ctr or CutC (***Section 2.10.3***). Each cDNA sample was diluted 1:10 with HPLC H_2O prior to QPCR and analysed in triplicate.

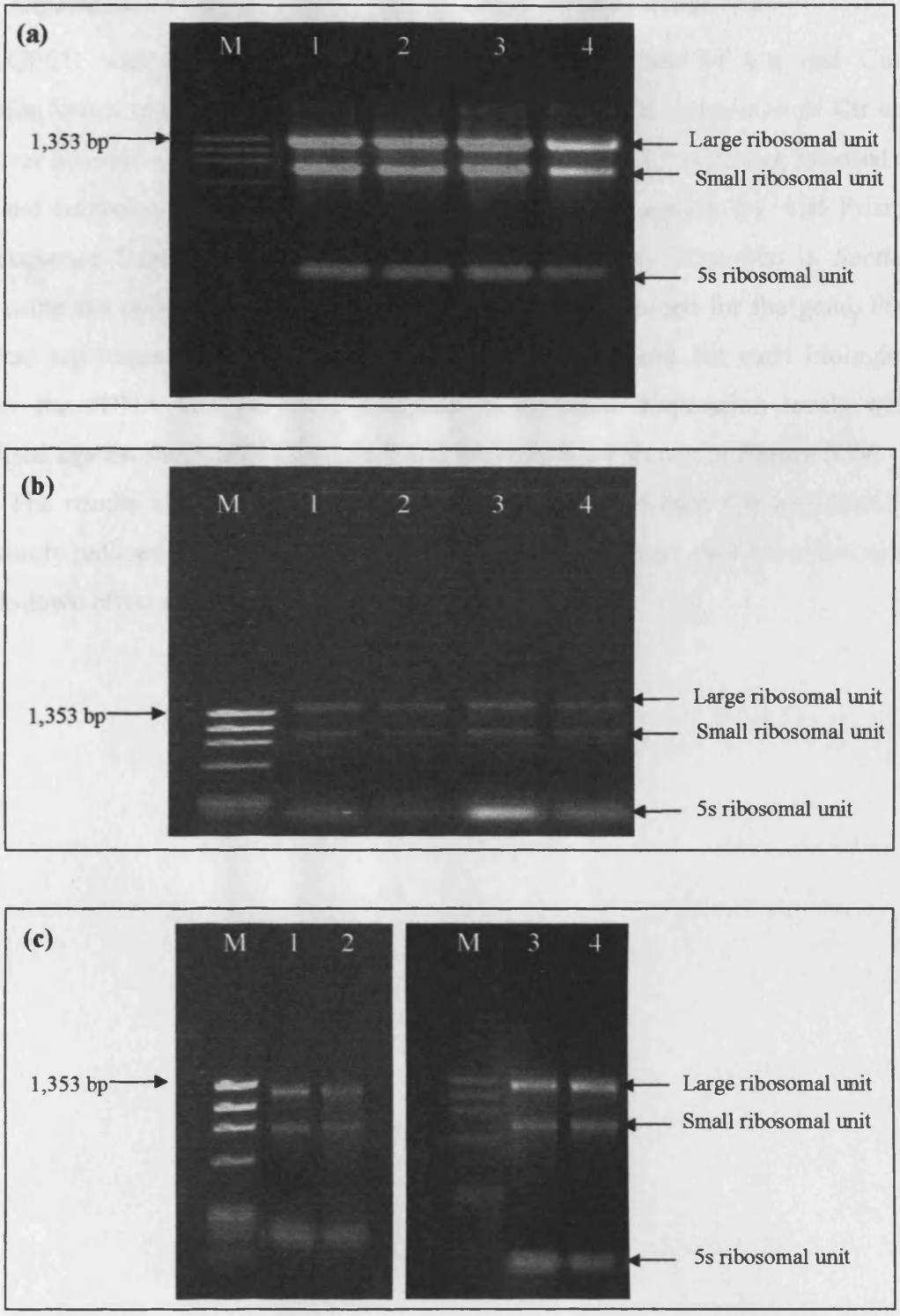

Figure 5.13 *Agarose gel electrophoresis of* C. elegans *RNA. Lane M: øX174H3 HaeIII digest marker, lane 1:* C. elegans *exposed to 0μM $CuSO_4$, lane 2: 20μM $CuSO_4$, lane 3: 100μM $CuSO_4$ and lane 4: 500μM $CuSO_4$. (a) control nematodes exposed to empty pPD129.36 vector (b) nematodes exposed to RNAi of Ctr and (c) nematodes exposed to RNAi of CutC.*

5.8.5 Expression of Ctr and CutC in C. elegans *exposed to RNAi*

QPCR was used to determine the knock-down effect of Ctr and CutC expression levels in *C. elegans* exposed to RNAi. The relative expression of Ctr and CutC over a range of $CuSO_4$ concentrations was compared in nematodes exposed to RNAi and control nematodes using TaqMan® probe technology on the ABI Prism® 7700 Sequence Detection System. QPCR was carried out as described in ***Section 2.11.6*** using the optimal primer and probe conditions determined for the gene. Five biological replicates were prepared for each test sample, and for each biological replicate the cDNA samples were analysed in triplicate. Expression levels were normalised against the control gene rpa-1 and the results are shown in ***Figure 5.14***.

The results show that relative level of expression of both Ctr and CutC is significantly reduced in *C. elegans* exposed to RNAi. This shows that RNAi did have a knock-down effect on the expression of the target gene.

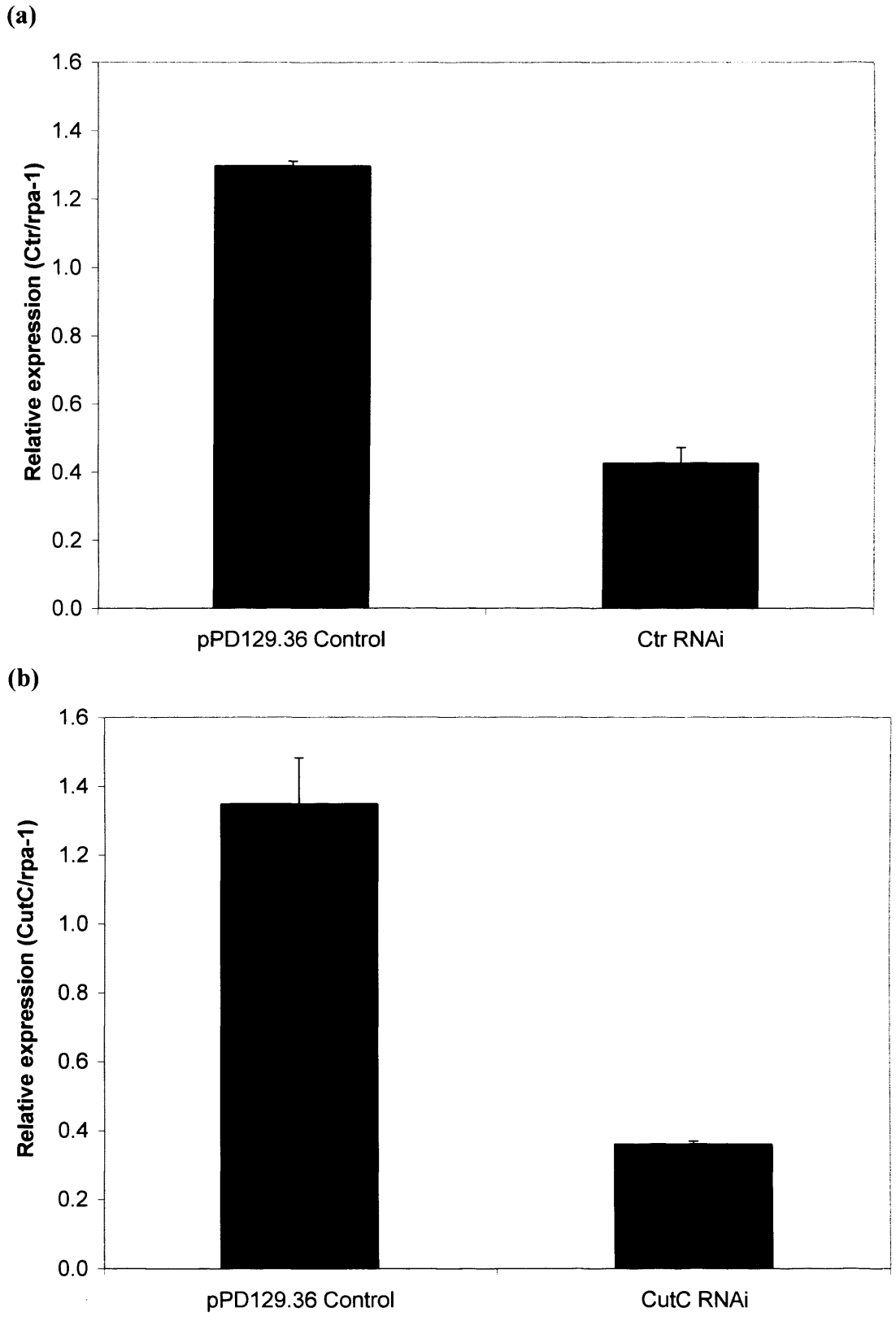

Figure 5.14 *The average relative expression of (a) Ctr and (b) CutC in control and RNAi exposed nematodes, ± SEM (n=5).*

5.9 Discussion

QPCR was used to determine the expression level of Ctr and CutC over different $CuSO_4$ concentrations. The results (***Figure 5.9***) showed that for both Ctr and CutC the relative gene expression is significantly down-regulated with increasing $CuSO_4$ concentrations. This suggests that the Ctr investigated may act as a Cu importer. At low Cu concentrations, Ctr actively imports the essential heavy metal in to the cell, but is down regulated in an environment with high levels of Cu, to prevent over accumulation of Cu and intracellular toxicity.

The CutC results were not as expected for an exporter or chaperone involved in Cu efflux; unless CutC is a high affinity chaperone, working under basal non-toxic conditions and a low affinity protein takes over when toxic levels of Cu are present in the environment.

RNAi was used to determine the functional significance of the genes by fully characterising life cycle parameters in *C. elegans* upon exposure to $CuSO_4$. Before performing the RNAi experiments, QPCR was used to validate the knock-down effect of RNAi. ***Figure 5.14*** showed that the expression of Ctr and CutC was significantly less in C. elegans exposed to RNAi. Thus with RNAi the use of exogenous Ctr or CutC interferes with the function of the endogenous target gene resulting in sequence specific silencing.

Chapter 6

Demographics of *C. elegans* exposed to RNAi

Chapter 6

Demographics of *C. elegans* exposed to RNAi

6.1 RNAi of Ctr and CutC

6.1.1 Introduction

The life cycle traits of nematodes exposed to RNAi were studied to determine the functional significance of Ctr and CutC. The function of the endogenous gene was interrupted using exogenous RNA and nematodes were exposed to a range of $CuSO_4$ concentrations to identify any changes in the life cycle traits compared to wild type *C. elegans*.

6.1.2 Experimental set up

Experiments were performed on NGM agar plates over a range of $CuSO_4$ concentrations (0μM to 500μM). The agar was seeded with the *E. coli* strain HT115 which contained the RNAi constructs as a food source for the *C. elegans*. For all experiments an egg preparation was performed to synchronise the *C. elegans* population before the exposure (***Section 2.3***). All experiments were performed at 20°C and the nematodes were transferred to fresh agar plates at appropriate intervals, with an appropriate number of replications as described.

6.2 Brood size

6.2.1 Total brood size

The number of progeny produced over a range of Cu concentrations was analysed with RNAi of the Ctr and CutC genes. An egg preparation (***Section 2.3***) was performed and individual L4 nematodes were placed on NGM agar plates. Every 36 hours the nematode was transferred to a fresh plate and the number of progeny

counted. For each test 20 individual replicates were performed. ***Figure 6.1*** shows the average number of progeny produced over a range of $CuSO_4$ concentrations.

The results show that in both the control nematodes and those exposed to RNAi of Ctr and CutC the number of progeny decreased with increasing $CuSO_4$ concentrations. In control nematodes at 0μM $CuSO_4$ around 180 progeny are produced in the total. This is lower than would normally be expected as nematodes are able to produce as many as 200-300 progeny in ideal conditions. This may be a result of increased handling and transferring of nematodes from plate to plate.

Based on an Anderson-Darling plot, all results are normally distributed; hence two-sample t-tests (2 tailed) were performed to determine the significant difference between the means. A statistical significant difference was identified by a p-value of less than 0.05.

For both control and test nematodes (with the exception of 0μM to 20μM $CuSO_4$ with CutC RNAi) a significant difference in the number of progeny was seen at all $CuSO_4$ concentrations ($p \leq 0.05$). At 100μM $CuSO_4$ control nematodes exposed to the empty RNAi vector (pPD129.36) showed a brood size reduction of 51% which effectively equates to the EC50 value.

At 0μM $CuSO_4$, RNAi of the Ctr gene resulted in a significant reduction of the brood size compared to control nematodes ($p \leq 0.009$). Though statistically insignificant, more progeny were produced by Ctr RNAi exposed nematodes on all plates containing $CuSO_4$.

At 20μM and 100μM $CuSO_4$ nematodes exposed to RNAi of CutC produced significantly more progeny than control nematodes ($p \leq 0.000$ and $p \leq 0.032$, respectively). Whilst not statistically significant 30% less progeny were produced at 500μM $CuSO_4$ in nematodes exposed to RNAi of CutC compared to control.

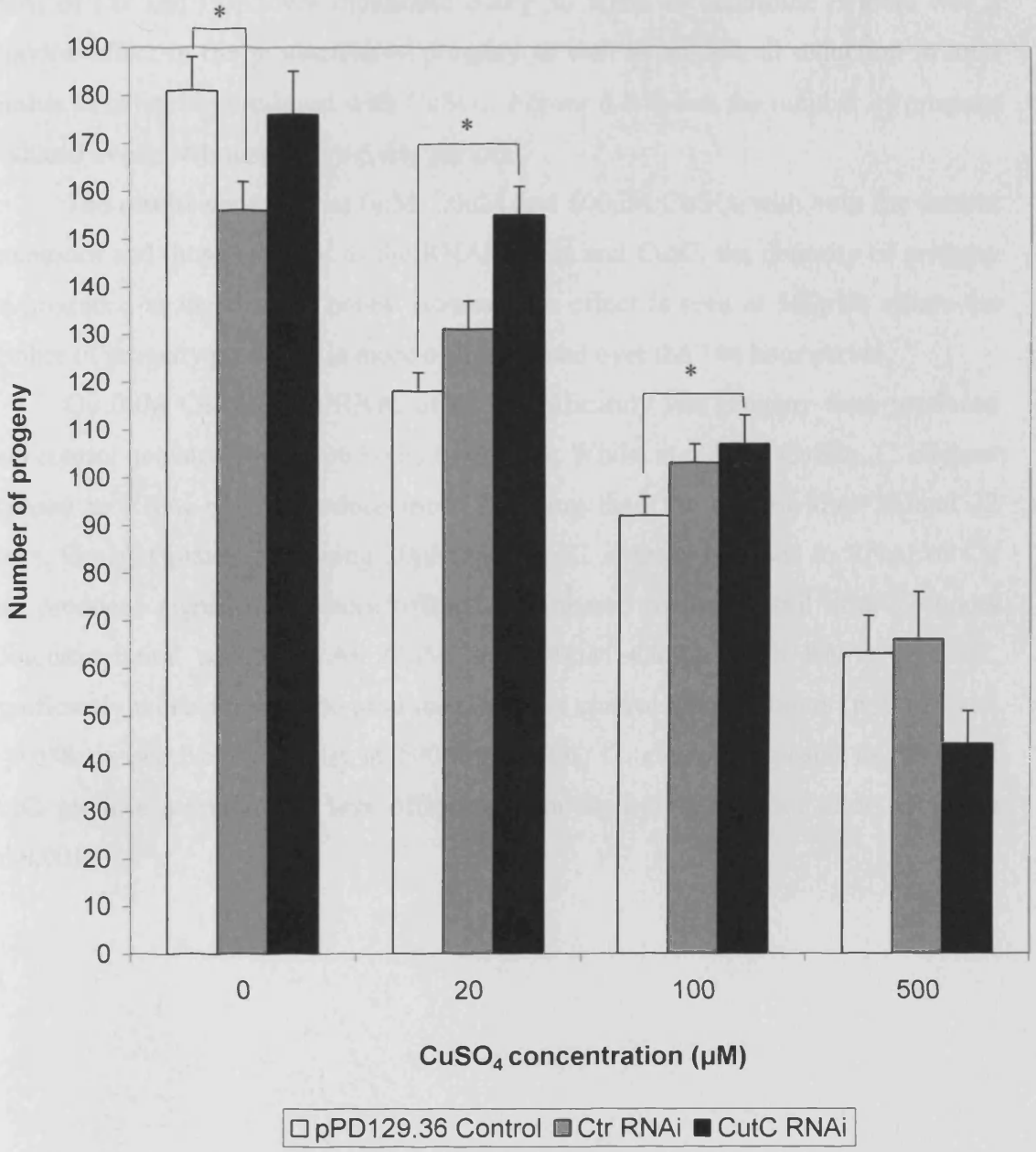

Figure 6.1 *The effect of different $CuSO_4$ concentrations on the total number of progeny produced with RNAi of Ctr and CutC in* C. elegans, ± *SEM (n=20). Significant differences between control and RNAi test are denoted by *.*

6.2.2 Temporal effect on brood number

The number of progeny produced by control nematodes and those exposed to RNAi of Ctr and CutC was monitored every 36 hours to determine if there was a temporal effect in the production of progeny as well as an overall reduction in total number of offspring produced with $CuSO_4$. ***Figure 6.2*** shows the number of progeny produced every 36 hours over a 6 day period.

The results show that at 0μM, 20μM and 100μM $CuSO_4$ with both the control nematodes and those exposed to the RNAi of Ctr and CutC, the majority of progeny are produced in the first 36 hours. However an effect is seen at 500μM, where the number of progeny produced is more evenly spread over the 144 hour period.

On 0μM $CuSO_4$ with RNAi of Ctr, significantly less progeny were produced than control nematodes after 36 hours ($p \leq 0.000$). Whilst at 500μM $CuSO_4$, *C. elegans* exposed to RNAi of Ctr produce more offspring than the control after 36 and 72 hours. On agar plates containing 20μM $CuSO_4$, *C. elegans* exposed to RNAi of Ctr also produced significantly more offspring compared to the control after 72 hours (Ubiquitinylation $p \leq 0.015$). At 20μM and 100μM $CuSO_4$, with RNAi of CutC, significantly more progeny are produced than the control after 36 hours ($p \leq 0.026$ and $p \leq 0.038$, respectively). Whilst at 500μM $CuSO_4$, *C. elegans* exposed to RNAi of CutC produce significantly less offspring than control nematodes after 36 hours ($p \leq 0.001$).

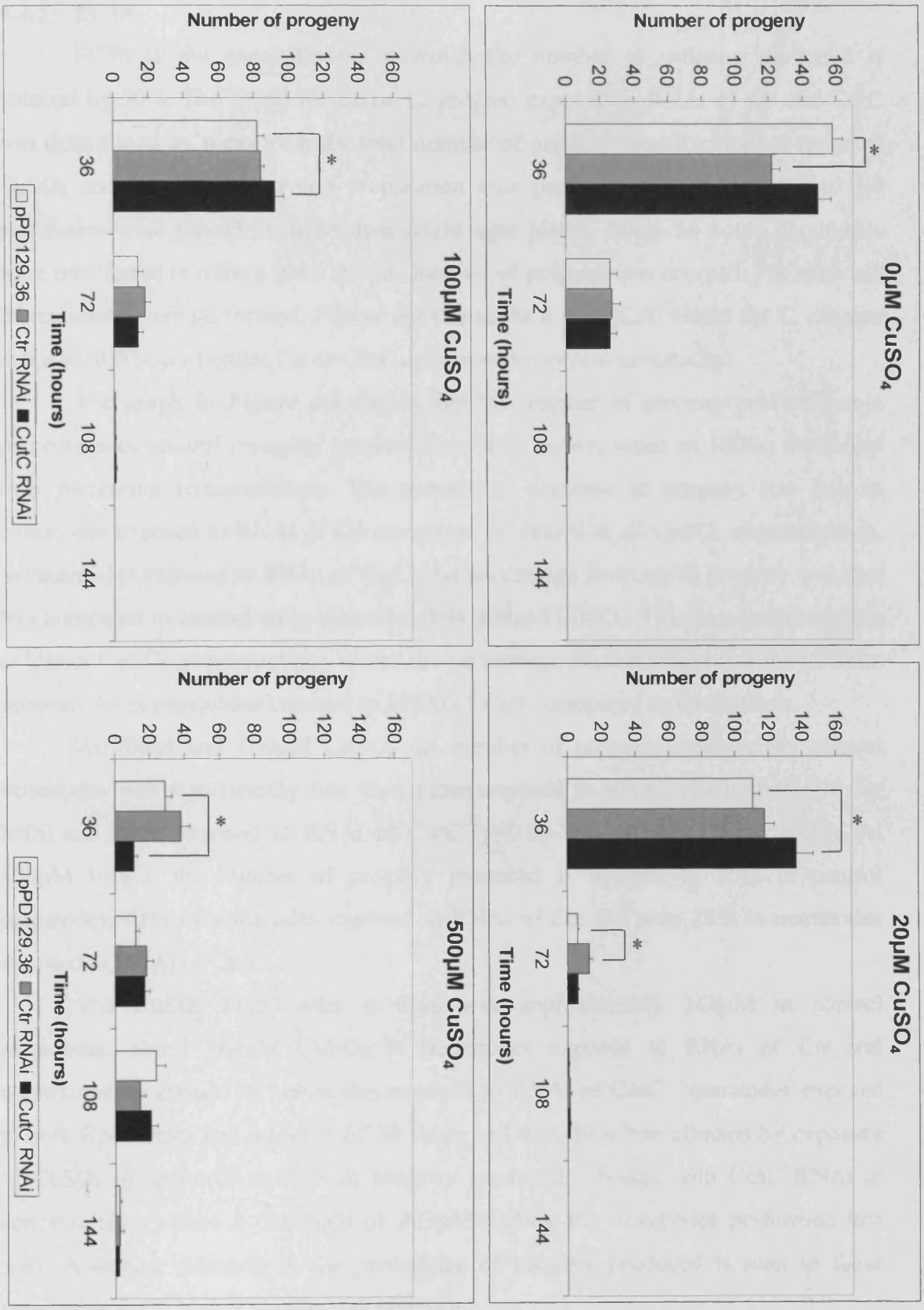

Figure 6.2 *Effect of $CuSO_4$ on brood size over 144 hours with RNAi of Ctr and CutC, ± SEM (n=20). Significant differences between control and RNAi exposed nematodes are denoted by *.*

6.2.3 EC50

EC50 is the concentration at which the number of progeny produced is reduced by 50%. The EC50 for Cu on *C. elegans* exposed to RNAi of Ctr and CutC was determined by measuring the total number of progeny produced over a range of $CuSO_4$ concentrations. An egg preparation was performed (***Section 2.3***) and L4 nematodes were placed on individual NGM agar plates. Every 36 hours nematodes were transferred to a fresh plate and the number of progeny was counted. For each test 20 replicates were performed. ***Figure 6.3*** shows the 6 day EC50 values for *C. elegans* exposed to RNAi of either Ctr or CutC compared to control nematodes.

The graph in ***Figure 6.3*** shows that the number of progeny produced as a percentage of control (progeny produced on 0μM $CuSO_4$ taken as 100%) decreased over increasing concentrations. The percentage decrease in progeny was less in nematodes exposed to RNAi of Ctr compared to control at all $CuSO_4$ concentrations. In nematodes exposed to RNAi of CutC, the percentage decrease in progeny was also less compared to control up to approximately 290μM $CuSO_4$. This then switched over at higher $CuSO_4$ concentrations where the percentage decrease in progeny was more pronounced in nematodes exposed to RNAi of CutC compared to the controls.

At 20μM and 100μM $CuSO_4$ the number of progeny produced by control nematodes was significantly less than those exposed to RNAi of Ctr ($p \leq 0.000$ for both) and those exposed to RNAi of CutC ($p \leq 0.00$ and $p \leq 0.012$, respectively). At 500μM $CuSO_4$ the number of progeny produced is reduced to 35% in control nematodes, 42% in nematodes exposed to RNAi of Ctr and only 25% in nematodes exposed to RNAi of CutC.

The $CuSO_4$ EC50 after 6 days was approximately 114μM in control nematodes, about 364μM $CuSO_4$ in nematodes exposed to RNAi of Ctr and approximately 206μM in nematodes exposed to RNAi of CutC. Nematodes exposed to both RNAi tests had a higher EC50 value and were thus less affected by exposure to $CuSO_4$ in terms of number of progeny produced. Though with CutC RNAi at concentrations above a threshold of 293μM $CuSO_4$ the nematodes performed less well. A steeper decrease in the percentage of progeny produced is seen in these nematodes at the higher $CuSO_4$ concentrations.

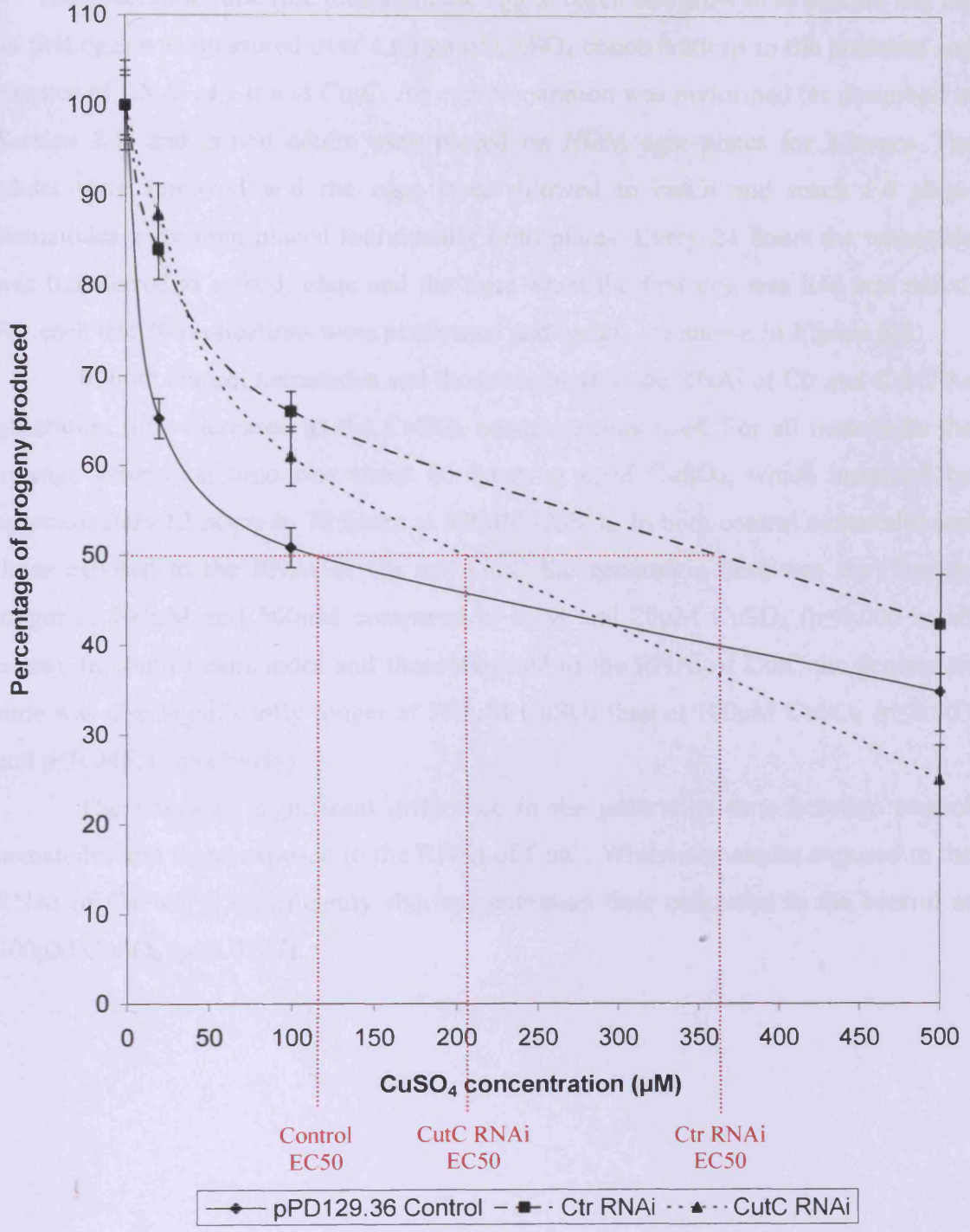

Figure 6.3 *Graph showing the percentage of progeny produced over different $CuSO_4$ concentrations, ± SEM (n=20). The EC50 value is indicated for control* C. elegans *and those exposed to RNAi of Ctr and CutC, ± SEM (n=20).*

6.3 Generation time

The generation time (the time from the egg to hatch and grow to adulthood and lay its first egg) was measured over a range of $CuSO_4$ concentrations in the presence and absence of RNAi of Ctr and CutC. An egg preparation was preformed (as described in ***Section 2.3***) and gravid adults were placed on NGM agar plates for 2 hours. The adults were removed and the eggs were allowed to hatch and reach L4 stage. Nematodes were then placed individually onto plates. Every 24 hours the nematode was transferred to a fresh plate and the time when the first egg was laid was noted. For each test 30 replications were performed and results are shown in ***Figure 6.4***.

In both control nematodes and those exposed to the RNAi of Ctr and CutC the generation time increased as the $CuSO_4$ concentrations rises. For all nematodes the average generation time was about 66 hours at 0μM $CuSO_4$, which increased by approximately 12 hours to 78 hours at 500μM $CuSO_4$. In both control nematodes and those exposed to the RNAi of Ctr and CutC the generation time was significantly longer at 100μM and 500μM compared to 0μM and 20μM $CuSO_4$ ($p \leq 0.000$ in all cases). In control nematodes and those exposed to the RNAi of CutC the generation time was also significantly longer at 500μM $CuSO_4$ than at 100μM $CuSO_4$ ($p \leq 0.003$ and $p \leq 0.045$, respectively).

There was no significant difference in the generation time between control nematodes and those exposed to the RNAi of CutC. Whilst nematodes exposed to the RNAi of Ctr had a significantly shorter generation time compared to the control at 500μM $CuSO_4$ ($p \leq 0.0227$).

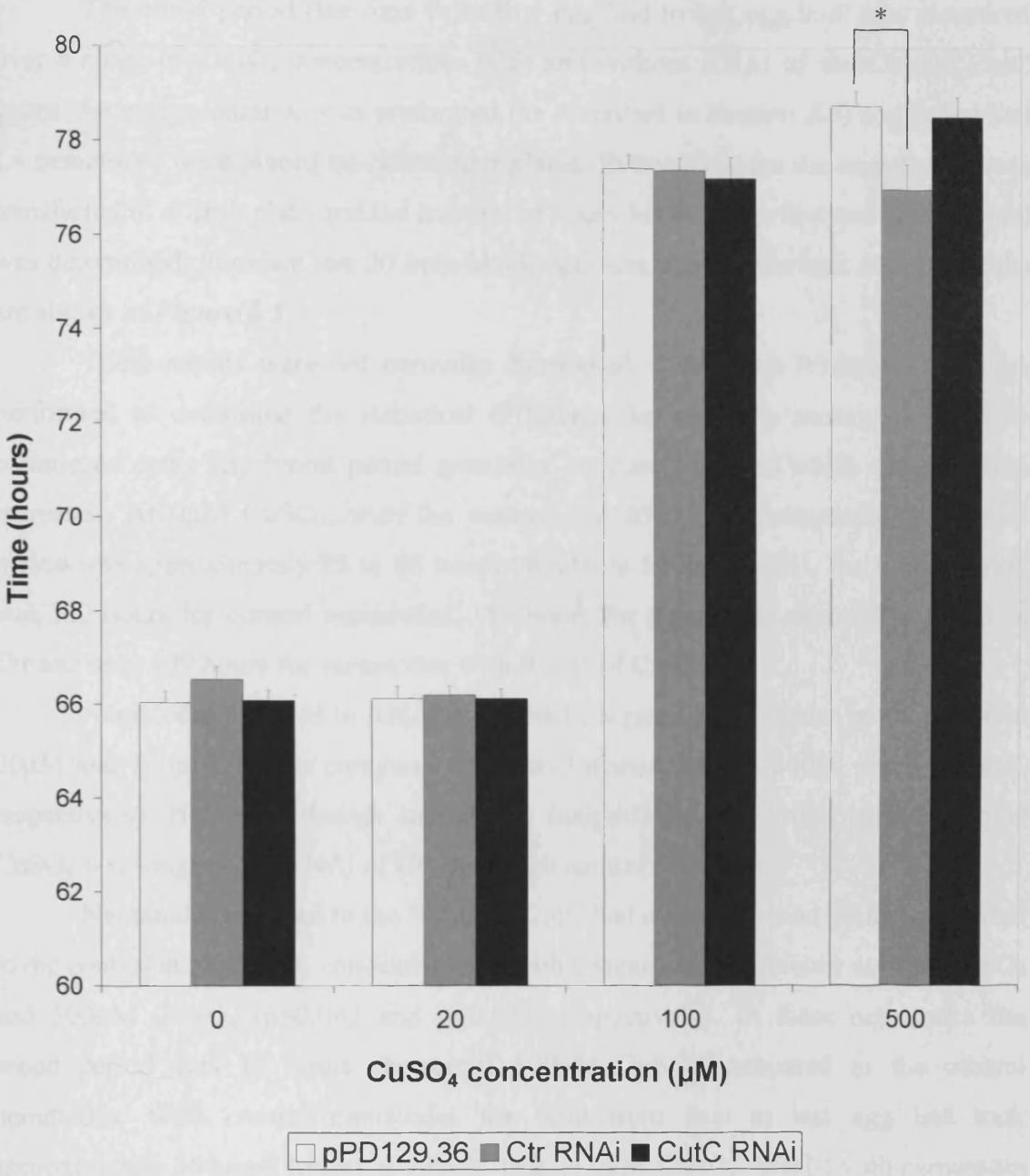

Figure 6.4 *Effect of $CuSO_4$ on the generation time of* C. elegans *exposed to RNAi of Ctr and CutC. Results shown as the time period from egg to egg, ± SEM (n=30). Significant differences between control and RNAi test are denoted by *.*

6.4 Brood period

The brood period (the time from first egg laid to last egg laid) was measured over a range of $CuSO_4$ concentrations with and without RNAi of the Ctr and CutC genes. An egg preparation was preformed (as described in ***Section 2.3***) and individual L4 nematodes were placed on NGM agar plates. Every 36 hours the nematodes were transferred to a fresh plate and the number of hours between the first and last egg laid was determined. For each test 20 individual replicates were performed and the results are shown in ***Figure 6.5***.

These results were not normally distributed, thus Mann-Whitney tests were performed to determine the statistical difference between the means of the non-parametric data. The brood period generally increased as the $CuSO_4$ concentration increased. At 0μM $CuSO_4$, with the control and RNAi test nematodes, the brood period was approximately 85 to 88 hours. Whilst at 500μM $CuSO_4$ the brood period was 122 hours for control nematodes, 113 hours for nematodes exposed to RNAi of Ctr and only 109 hours for nematodes with RNAi of CutC.

Nematodes exposed to RNAi of Ctr had a significantly shorter brood period at 20μM and 100μM $CuSO_4$ compared to control nematodes ($p \leq 0.0020$ and $p \leq 0.0412$, respectively). However, though statistically insignificant, the brood period at 0μM $CuSO_4$ was longer with RNAi of Ctr than with control nematodes.

Nematodes exposed to the RNAi of CutC had a shorter brood period compared to the control at all $CuSO_4$ concentrations, with a significant difference at 0μM $CuSO_4$ and 500μM $CuSO_4$ ($p \leq 0.042$ and $p \leq 0.038$, respectively). In these nematodes the brood period was 13 hours shorter at 500μM $CuSO_4$ compared to the control nematodes. With control nematodes the time from first to last egg laid took approximately 36 hours longer at 500μM than at 0μM $CuSO_4$. Whilst with nematodes exposed to the RNAi of CutC, the brood period was only about 24 hours longer at 500μM $CuSO_4$ compared to 0μM $CuSO_4$.

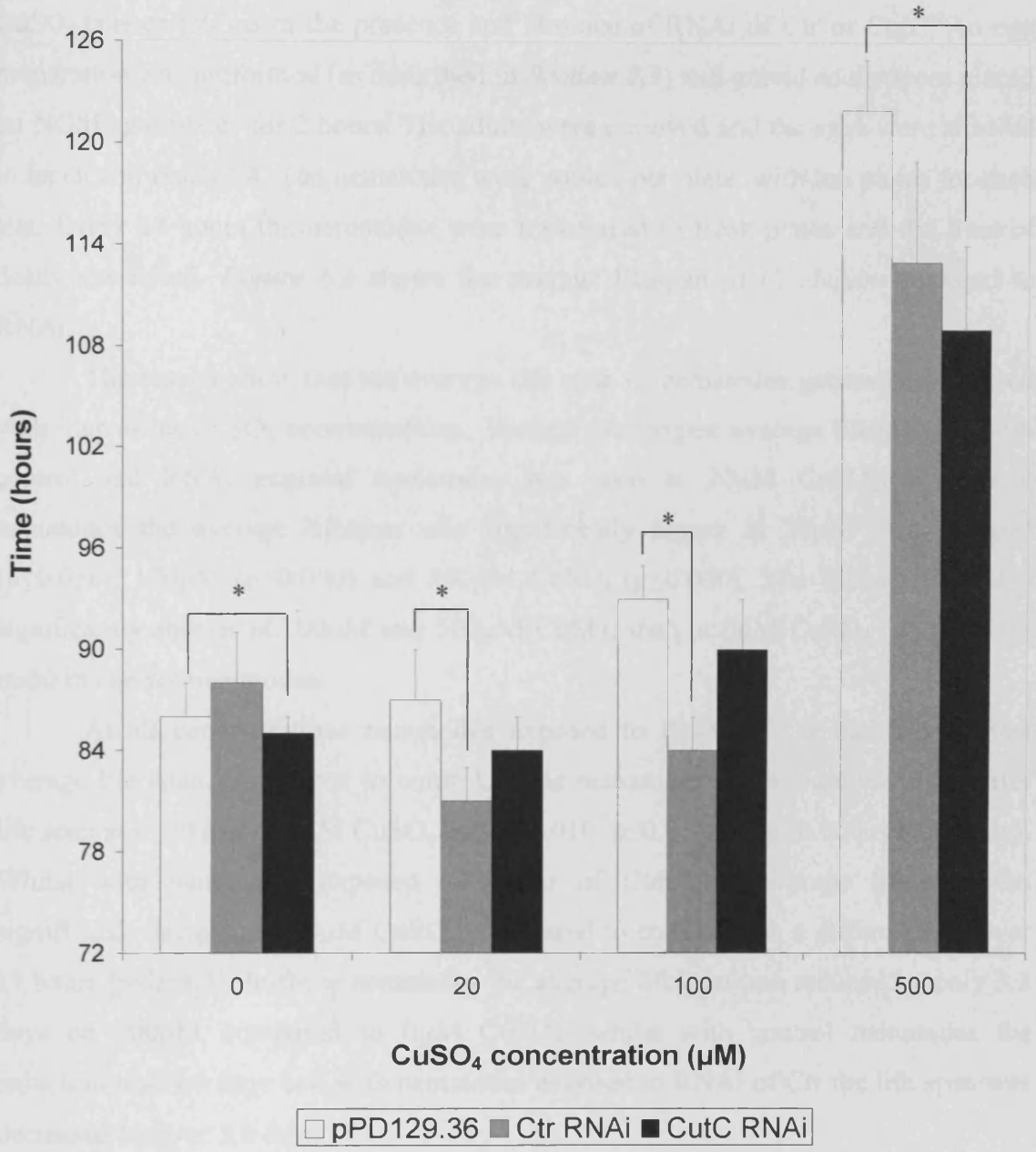

Figure 6.5 *Effect of different $CuSO_4$ concentrations on the average brood period of* C. elegans *with and without exposure to RNAi of Ctr anf CutC. Results are presented as average hours at 20°C, ± SEM (n=20). Significant differences between control and RNAi test are denoted by *.*

6.5 Life span

6.5.1 Average life span

The life span of the nematodes (egg to death) was measured over a range of $CuSO_4$ concentrations in the presence and absence of RNAi of Ctr or CutC. An egg preparation was preformed (as described in ***Section 2.3***) and gravid adults were placed on NGM agar plates for 2 hours. The adults were removed and the eggs were allowed to hatch and reach L4. Ten nematodes were pooled per plate, with ten plates for each test. Every 24 hours the nematodes were transferred to fresh plates and the time of death was noted. ***Figure 6.6*** shows the average lifespan of *C. elegans* exposed to RNAi.

The results show that the average life span of nematodes generally decreased with increasing $CuSO_4$ concentrations. Though the longest average lifespan for both control and RNAi exposed nematodes was seen at 20μM $CuSO_4$. In control nematodes the average lifespan was significantly longer at 20μM than at 0μM ($p \leq 0.016$), 100μM ($p \leq 0.000$) and 500μM $CuSO_4$ ($p \leq 0.000$). The lifespan was also significantly shorter at 100μM and 500μM $CuSO_4$ than at 0μM $CuSO_4$ ($p \leq 0.000$ for both) in control nematodes.

At all concentrations nematodes exposed to RNAi of Ctr had the shortest average life span. Compared to control, these nematodes had a significantly shorter life span at 0, 20 and 500μM $CuSO_4$ ($p \leq$ of 0.010, $p \leq 0.016$ and $p \leq 0.000$ respectively). Whilst with nematodes exposed to RNAi of CutC the average lifespan was significantly longer at 500μM $CuSO_4$, compared to control with a difference of over 17 hours ($p \leq 0.002$). In these nematodes the average lifespan was reduced by only 3.3 days on 500μM compared to 0μM $CuSO_4$, whilst with control nematodes the reduction was 4.6 days and with nematodes exposed to RNAi of Ctr the life span was decreased by over 5.6 days.

With both RNAi of Ctr and CutC the life span was significantly shorter at 500μM $CuSO_4$ than at 0μM, 20μM and 100μM $CuSO_4$ ($p \leq 0.000$ for all). There was also a significant decrease in the life span from 0μM to 100μM $CuSO_4$ ($p \leq 0.000$ for both) and from 20μM to 100μM $CuSO_4$ ($p \leq 0.000$ for both).

There was little difference in the minimum life span between the control and test nematodes and over different $CuSO_4$ concentrations. However, the maximum life span varied considerably both over the range of $CuSO_4$ concentrations and between

control nematodes and those exposed to RNAi. For both the control nematodes and those exposed to RNAi of Ctr and CutC, the longest maximum lifespan was seen at 20μM $CuSO_4$ (28 days). At 500μM $CuSO_4$ the maximum life span was shortest in nematodes exposed to RNAi of Ctr and was only 10 days, compared to 15 days with control nematodes. The reduction in the maximum life span on 500μM $CuSO_4$ compared to 0μM $CuSO_4$ was only 6 days for control nematodes, as much as 12 days with Ctr RNAi and 8 days with CutC RNAi.

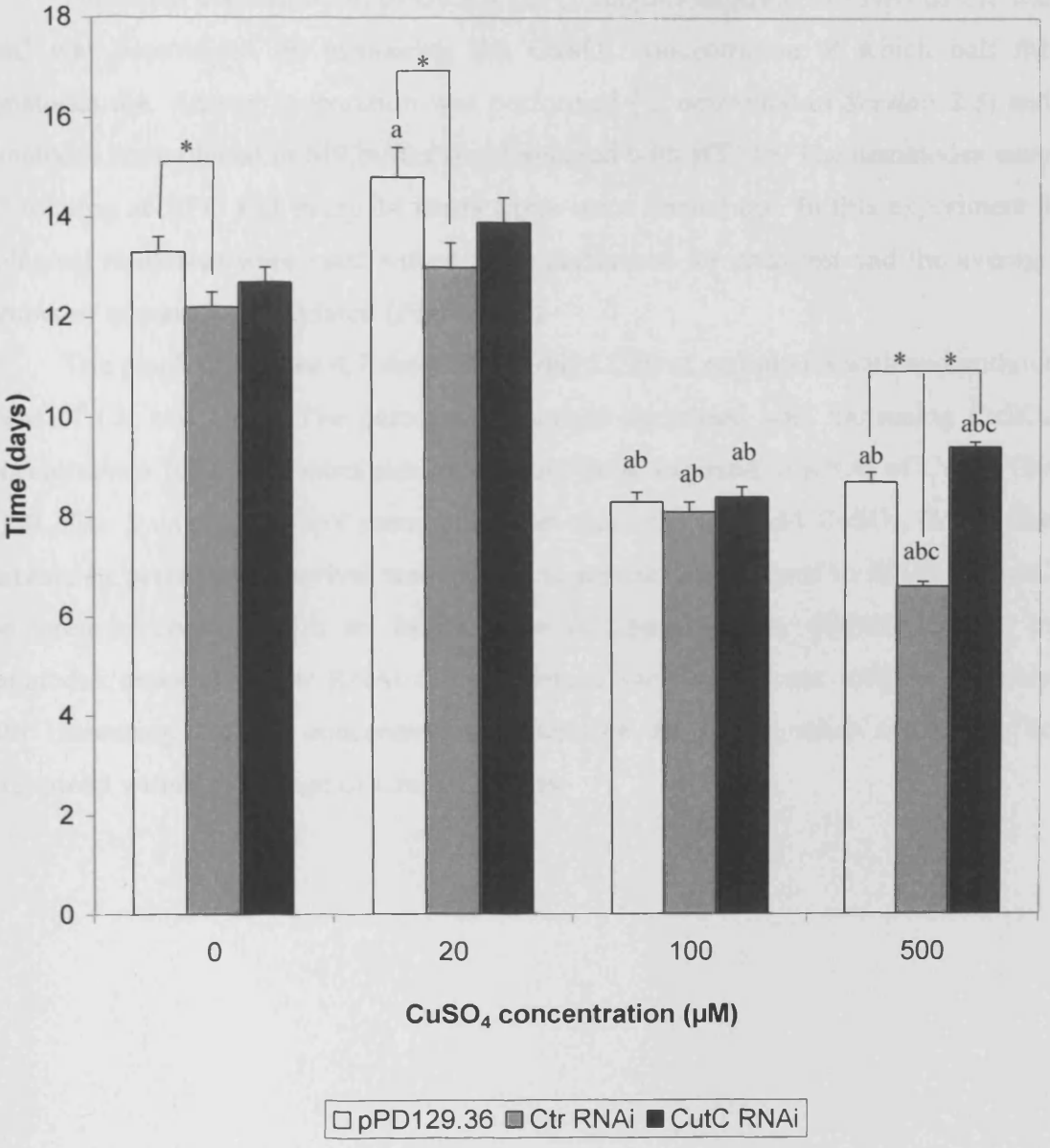

Figure 6.6 *Effect of different $CuSO_4$ concentrations on the life span of* C. elegans *with RNAi of Ctr and CutC. Results shown as average, life span of nematodes over a range, ± SEM (n=100).* * *denotes significant differences between control and RNAi test.* [a] *denotes a significant difference from 0µM $CuSO_4$,* [b] *denotes a significant difference from 20µM $CuSO_4$ and* [c] *denotes a significant difference from 100µM $CuSO_4$.*

6.5.2 LC50 of nematodes exposed to RNAi

The lethal concentration of $CuSO_4$ on *C. elegans* exposed to RNAi of Ctr and CutC was determined by measuring the $CuSO_4$ concentration at which half the nematodes die. An egg preparation was performed (as described in ***Section 2.3***) and nematodes were placed in M9 buffer supplemented with HT115. The nematodes were left rotating at 20°C and every 24 hours titres were carried out. In this experiment 3 biological replicates were used with 4 titres performed for each test and the average number of nematodes calculated (***Figure 6.7***).

The graph in ***Figure 6.7*** shows the 3 day LC50 of nematodes with and without RNAi of Ctr and CutC. The percentage survival decreased with increasing $CuSO_4$ concentrations for both control nematodes and those exposed to RNAi of CutC. The LC50 after 3 days in control nematodes was more than 700μM $CuSO_4$. Whilst the decrease in percentage survival was greater in nematodes exposed to RNAi of CutC compared to control, with an LC50 value of approximately 600μM $CuSO_4$. In nematodes exposed to Ctr RNAi the percentage survival did not actually decrease with increasing $CuSO_4$ concentrations, therefore an LC50 value could not be determined within this range of concentrations.

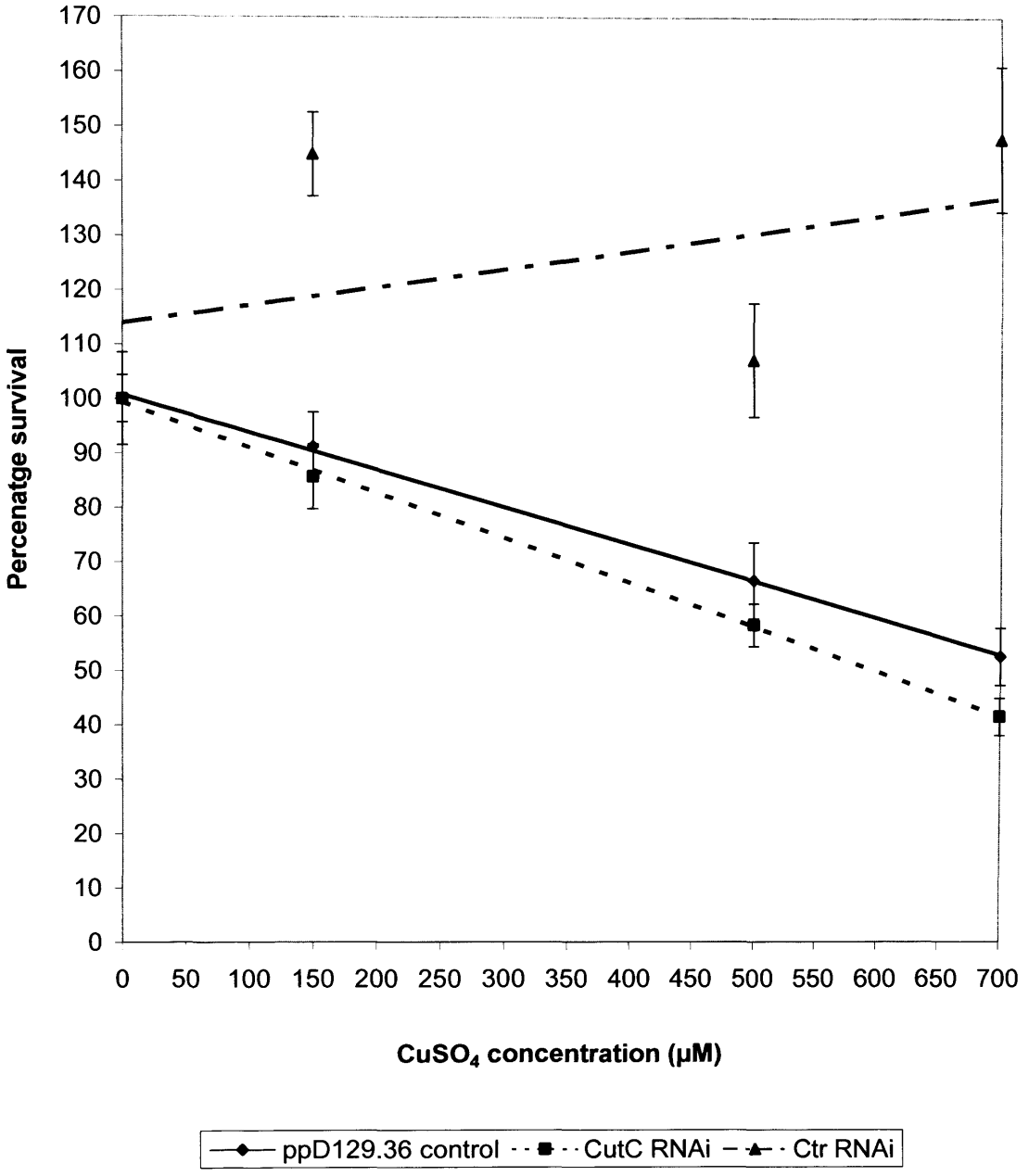

Figure 6.7 *The effect of $CuSO_4$ on the percentage survival of nematodes in liquid culture with RNAi of Ctr and CutC, ± SEM (n=3).*

6.6 Discussion

In all experiments, $CuSO_4$ had significant detrimental consequences on the demographics of *C. elegans* with and without RNAi of Ctr and CutC. Overall the general toxic effect of Cu on *C. elegans* was a reduction in brood size and life span and an increase in the brood period and generation time.

For both control and RNAi test nematodes the brood size was significantly decreased at each increasing concentration of $CuSO_4$ (with the exception of CutC RNAi from 0μM to 20μM $CuSO_4$). Therefore elevated levels of Cu had a detrimental effect on nematodes in terms of the number of progeny produced.

At 0μM $CuSO_4$, nematodes with RNAi of the putative Ctr gene produced significantly less progeny than the control. This indicates that the knock down of Ctr gene influences the production of progeny. Cu is an essential metal required for embryogenesis and development, thus RNAi of the Ctr gene may have resulted in insufficient basal Cu uptake at 0μM $CuSO_4$, causing a reduction in brood size. Recent studies with Ctr1 KO mice have also highlighted an essential function in mammalian embryonic development since homozygous mutants die *in utero* (Sharp, 2003). As this result is seen at basal levels of $CuSO_4$, it indicates that Ctr may have a role in high affinity Cu uptake. A low affinity importer would function under conditions where Cu concentrations were sufficient. Whilst a high affinity transporter would be expressed when extracellular Cu concentrations were limited and active import was required. As effects of RNAi of Ctr are seen at 0μM $CuSO_4$ it indicates that this Ctr has a role under limiting Cu conditions and is therefore likely to be a high affinity transporter.

At 20μM and 100μM nematodes exposed to RNAi of CutC produced significantly more offspring than control, but 30% less offspring at 500μM $CuSO_4$. Therefore nematodes exposed to RNAi of CutC perform better at lower $CuSO_4$ concentrations but less well at higher concentrations. This indicates that CutC may be involved in the efflux of Cu from the cell or in intracellular storage of free Cu ions, as when the function of the gene is disrupted by RNAi the nematodes are less able to tolerate elevated Cu concentrations. Previous studies have shown that in *E. coli* the CutC mutant is Cu sensitive and accumulates Cu but has apparently normal kinetics of Cu uptake. Thus the CutC gene was postulated to encode for an efflux protein which removes excess Cu from the cytoplasm (Gupta *et al.*, 1995).

$CuSO_4$ also appears to have an effect on the production of progeny in a temporal manner. At 0μM, 20μM and 100μM $CuSO_4$ with both the control nematodes and those exposed to the RNAi of Ctr and CutC, the majority of progeny are produced in the first 36 hours. Whilst at 500μM $CuSO_4$ a greater number of progeny are produced later and some even 6 days after reaching adulthood compared to the maximum 3 days without Cu.

Nematodes exposed to RNAi of CutC produced significantly more offspring compared to control at 20μM and 100μM $CuSO_4$ after 36 hours but significantly less at 500μM $CuSO_4$. This may be due to high Cu concentrations slowing down the development of nematodes exposed to RNAi of CutC as well as a direct detrimental effect on the number of progeny produced.

The $CuSO_4$ EC50 after 6 days was approximately 114μM in control nematodes and about 364μM $CuSO_4$ in nematodes exposed to RNAi of Ctr. Therefore nematodes exposed to RNAi of Ctr have a higher EC50 value and are thus less affected by exposure to $CuSO_4$ in terms of number of progeny produced. This further substantiates the notion that Ctr is a Cu importer. With exposure to RNAi the importers function has been disrupted therefore less Cu enters the cell and the nematodes are able to perform better at higher Cu concentrations.

With RNAi of CutC the $CuSO_4$ EC50 after 6 days was approximately 200μM, which was also higher compared to control nematodes but at concentrations above a threshold concentration of 293μM $CuSO_4$. Cu proves to be more toxic to the RNAi exposed nematodes than to controls, indicating that CutC has a complex role in Cu homeostasis.

The generation time was significantly increased at 100μM and 500μM $CuSO_4$, in both the control and RNAi exposed nematodes, indicating that toxic levels of Cu has a detrimental effect on development in *C. elegans*. Though nematodes exposed to the RNAi of Ctr had a significantly shorter generation time than control nematodes at 500μM $CuSO_4$. This may be because the function of the Cu transporter is disrupted, so less Cu is imported into the cell allowing the nematode to perform better at higher Cu concentrations. The brood period also generally increased with rising concentrations of $CuSO_4$ and was significantly longer at 500μM $CuSO_4$ in both control and RNAi exposed nematodes.

Elevated levels of $CuSO_4$ had a significant detrimental effect on the life span of *C. elegans* with both the control and RNAi tests. This is probably due to the fact that excess intracellular Cu causes the production of free radicals which leads to oxidative damage causing aging and death (***Section 1.12***).

In control nematodes and those exposed to CutC RNAi the percentage survival decreased with increasing $CuSO_4$ concentrations. The LC50 after 3 days was approximately 610μM $CuSO_4$ in nematodes exposed to RNAi of CutC but greater than 700μM $CuSO_4$ in control nematodes. Thus nematodes exposed to RNAi of CutC have a lower LC50 value and are less able to survive at elevated concentrations of $CuSO_4$, indicating a role for CutC in Cu efflux or intracellular storage.

With nematodes exposed to Ctr RNAi though, the percentage survival did not actually decrease at all with increasing $CuSO_4$ concentration up to 700μM $CuSO_4$. This is consistent with the hypothesis that the putative Ctr is a Cu transporter in *C. elegans*. The RNAi disrupts the function of the Cu importer therefore less Cu is imported into the cell allowing the nematode to survive at higher Cu concentrations.

Chapter 7

Phenotypic effects of *C. elegans* exposed to RNAi

Chapter 7

Phenotypic effects of *C. elegans* exposed to RNAi

7.1 Effect of copper on the growth and development of *C. elegans*

7.1.1 *Effect of copper on the growth of* C. elegans *with RNAi of Ctr and CutC*

During the toxicity experiments it was observed that nematodes exposed to $CuSO_4$ were smaller and grew at a slower rate. Therefore studies were carried out to determine the size and growth rate of nematodes on $CuSO_4$ plates with RNAi of Ctr and CutC compared to control.

An egg preparation was performed (***Section 2.3***) to stage the nematodes. L1s were placed onto agar plates containing various concentrations of $CuSO_4$ with and without exposure to RNAi. At least 15 individuals were analysed and nematodes were photographed every 24 hours over 5 days. ***Figure 7.1*** shows sample photographs from the experiment.

The photographs in ***Figure 7.1*** show that the size of nematodes decreased with increasing $CuSO_4$ concentrations with both control and RNAi exposures. In particular at 100μM and 500μM $CuSO_4$ the nematodes exposed to RNAi of CutC were considerably smaller than control nematodes. Using the Image Pro Express software the length and volume of nematodes was determined. The volume of the nematode was calculated using the equation below and the results are shown in ***Figure 7.2***

$$\text{Volume} = \frac{0.125 \times 3.1416 \times \text{Area}^2}{0.5 \times \text{length}}$$

Equation 1 Calculation to determine the volume of nematodes from the length and area.

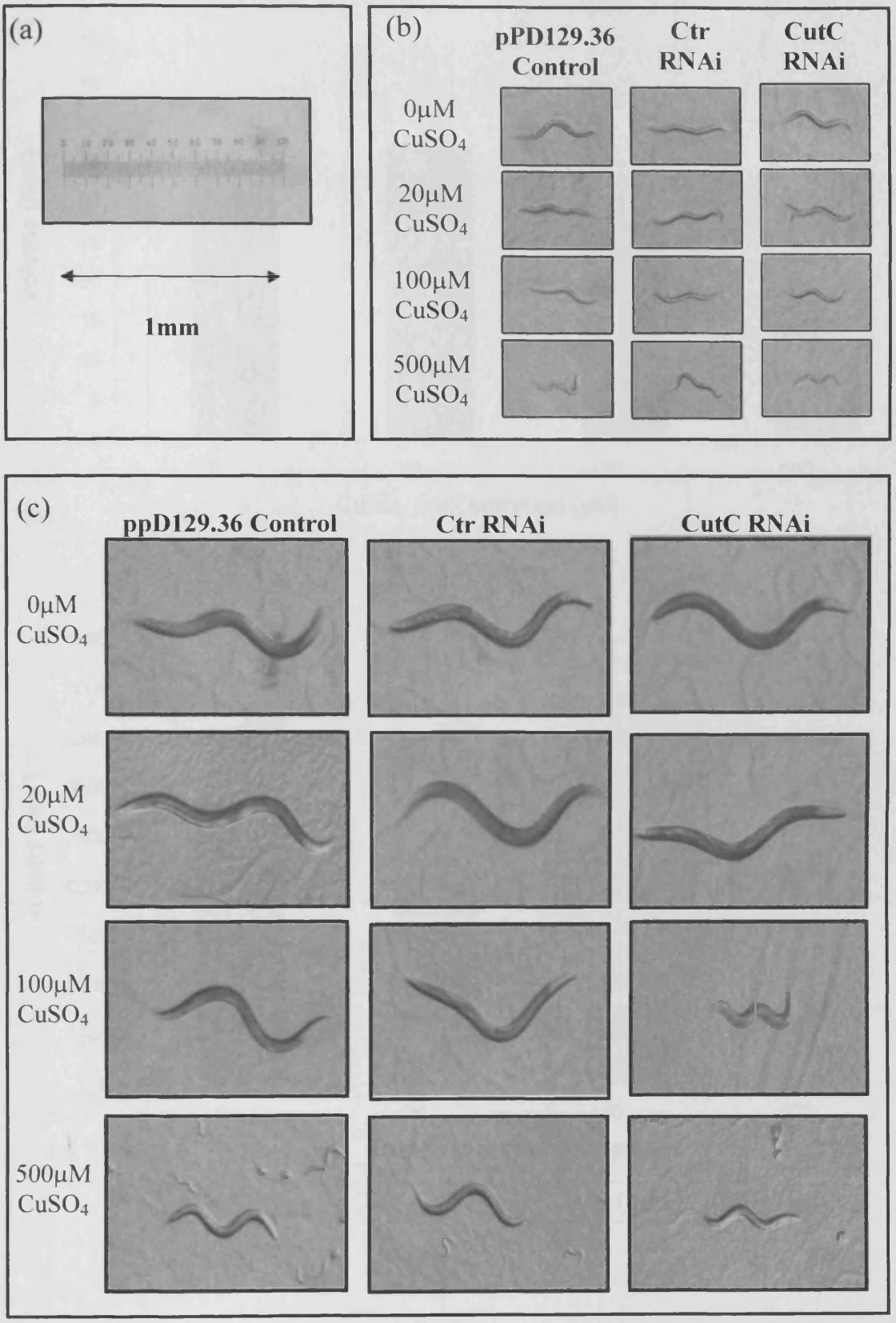

Figure 7.1 *Photographs showing the effect of $CuSO_4$ on the size of* C. elegans *with and without RNAi. (a) scale (b)1 day old stage L1 nematodes (c) 5 day old adult nematodes.*

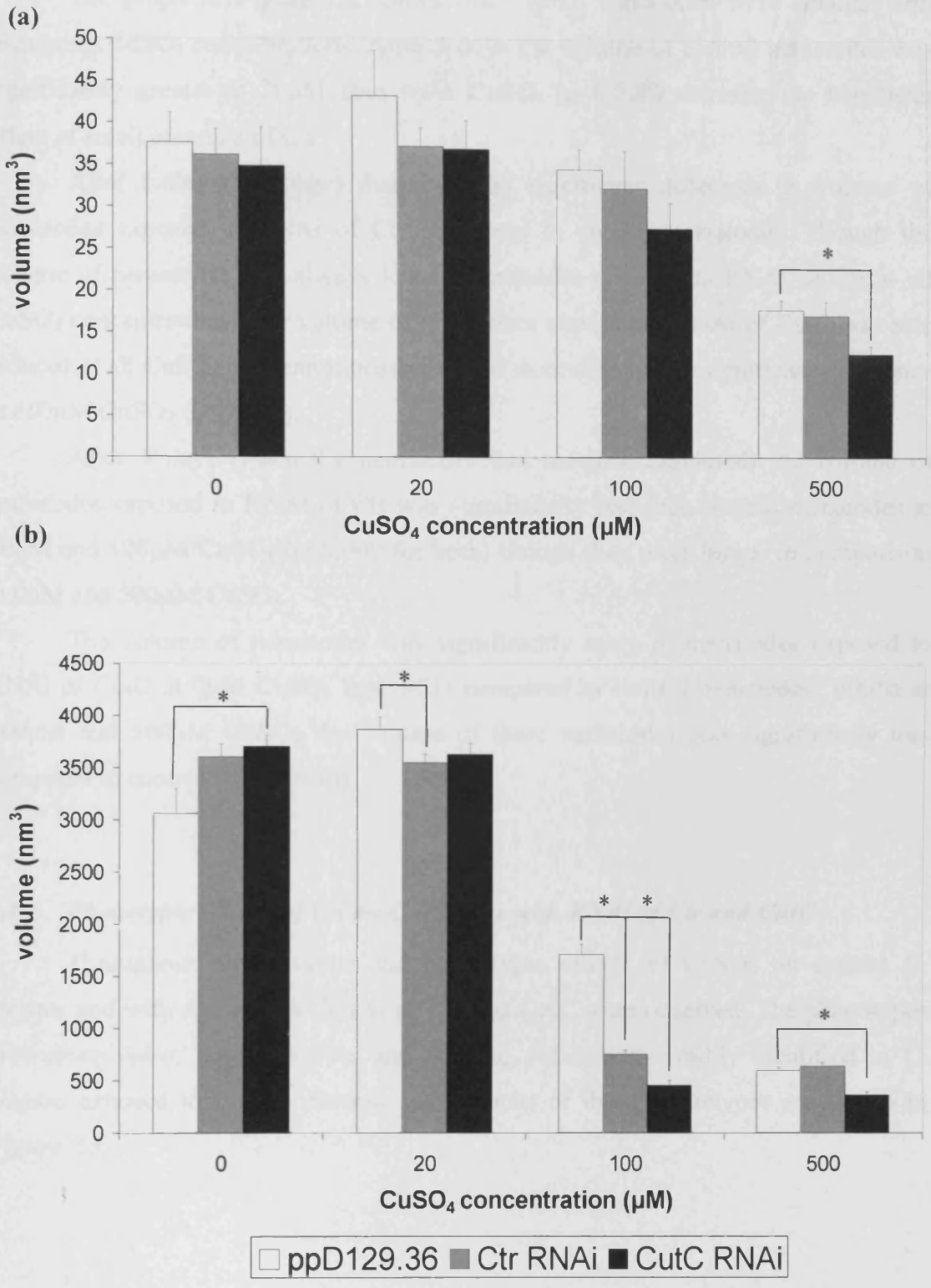

Figure 7.2 *The volume of nematodes over different $CuSO_4$ concentrations after (a) 1 day and (b) 5 days with RNAi of Ctr and CutC, ± SEM (n=15).*

The graph in ***Figure 7.2*** shows that overall nematodes were smaller with increasing $CuSO_4$ concentrations. After 5 days the volume of control nematodes was significantly greater at 20μM than 0μM $CuSO_4$ ($p \leq 0.006$) showing the beneficial effect of small amounts of Cu.

After 1 day (L1 stage) there was no significant difference in volume of nematodes exposed to RNAi of Ctr compared to control nematodes. Though the volume of nematodes was always less in nematodes exposed to RNAi of Ctr at all $CuSO_4$ concentrations. The volume of nematodes exposed to RNAi of CutC was also reduced at all $CuSO_4$ concentrations compared to control with a significant difference at 500μM $CuSO_4$ ($p \leq 0.003$).

After 5 days (when the nematodes had reached adulthood) the volume of nematodes exposed to RNAi of Ctr was significantly less then control nematodes at 20μM and 100μM $CuSO_4$ ($p \leq 0.000$ for both) though they were bigger in comparison at 0μM and 500μM $CuSO_4$.

The volume of nematodes was significantly more in nematodes exposed to RNAi of CutC at 0μM $CuSO_4$ ($p \leq 0.021$) compared to control nematodes. Whilst at 100μM and 500μM $CuSO_4$ the volume of these nematodes was significantly less compared to control ($p \leq$ for both).

7.1.2 *Phenotypic effects of Cu on C. elegans with RNAi of Ctr and CutC*

Throughout these studies the phenotypic effects of $CuSO_4$ on control *C. elegans* and with exposure to RNAi of Ctr and CutC were observed. The phenotypes *protruding vulva*, *bag of worms* and *molting defect* were readily identified in *C. elegans* exposed to $CuSO_4$. Sample photographs of these phenotypes are shown in ***Figure 7.3***.

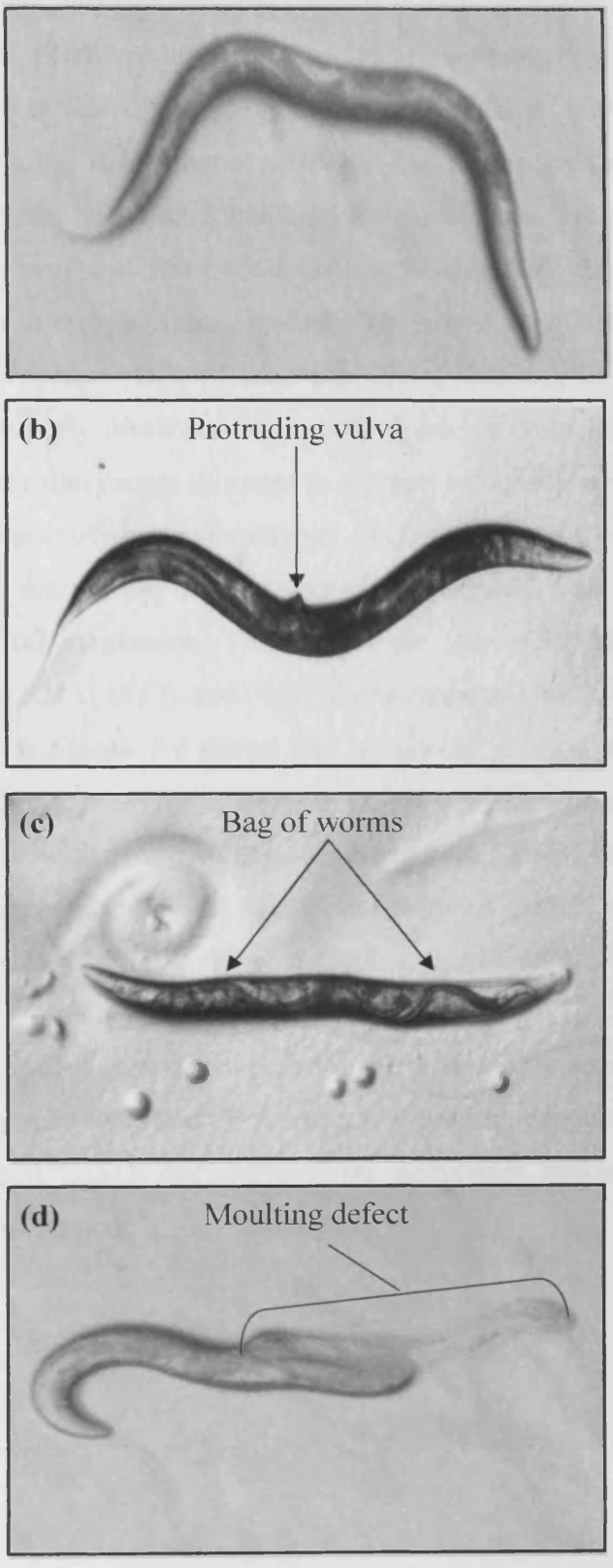

Figure 7.3 *Phenotypic effects of* C. elegans *on 500µM $CuSO_4$. (a) control (wild-type) nematode, (b) protruding vulva, (c) bag of worms and (d) moulting defect.*

The phenotype, *protruding vulva* (***Figure 7.3(b)***) is where the vulva protrudes from the nematode, often resulting in egg laying defects. *Bag of worms* (***Figure 7.3(c)***) is also a defect where the vulva has not developed properly and is often seen alongside the *protruding vulva* phenotype. With this phenotype the adult is unable to lay eggs, which results in the eggs hatching inside the adult; the young continue to grow and eat their way out from the inside until the adult dies and disintegrates allowing the young to escape. *Moulting defect* (***Figure 7.3(c)***) is where the nematode moults at an inappropriate stage of their life cycle, resulting in a defect where the outer skin is not properly discarded. As moulting occurs from the midsection of the nematode the defect often causes this area to rupture, killing the nematode.

The prevalence of these phenotypes over a range of $CuSO_4$ concentrations were observed and the number of nematodes that perished from *bag of worms* was determined from 100 nematodes. The results are shown in ***Figure 7.4*** with and without exposure to RNAi of Ctr and CutC over a range of $CuSO_4$ concentrations.

The results in ***Figure 7.4*** shows that nematodes exposed to RNAi of Ctr and CutC displayed the abnormal phenotype at all $CuSO_4$ concentrations with an increasing incidence with rising $CuSO_4$ concentrations. Those exposed to RNAi of CutC had the highest percentage of *bag of worms* at all $CuSO_4$ concentrations, with 15% of the nematodes displaying the phenotype at 0μM $CuSO_4$, increasing to 74% at 500μM $CuSO_4$.

Whilst in control nematodes the abnormality was only seen at 100μM $CuSO_4$ and 500μM $CuSO_4$, with 10% and 70% respectively of the nematodes developing *bag of worms*. At 500μM $CuSO_4$ only 50% of the nematodes exposed to RNAi of Ctr perished from bag of worms.

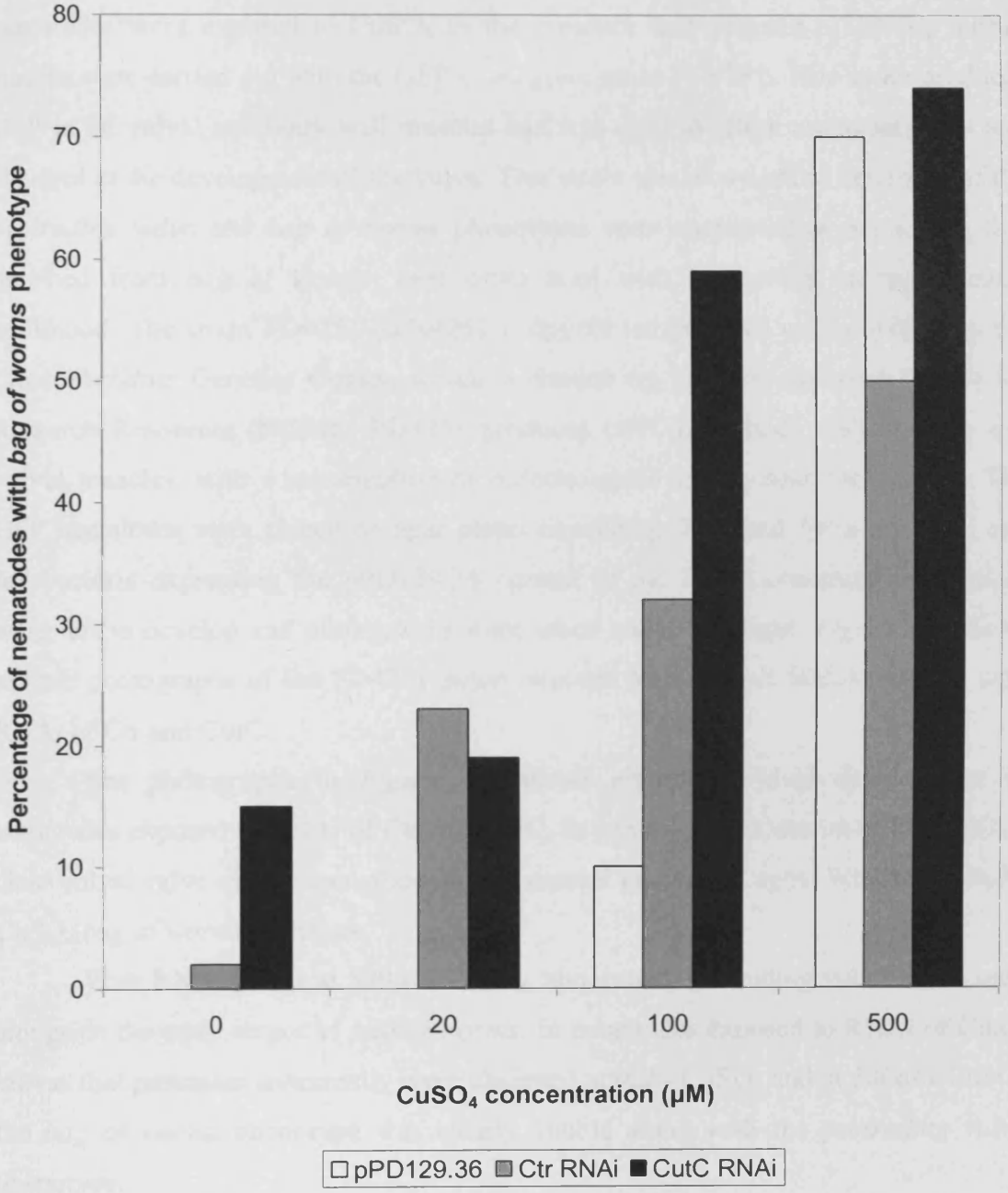

Figure 7.4 *Percentage of nematodes with the* bag of worms *phenotype over different $CuSO_4$ concentrations with and without RNAi of Ctr and CutC, n=100.*

7.2 Vulva Green Fluorescence protein (GFP)

As numerous defects were seen in the development of the vulva when nematodes were exposed to $CuSO_4$ in the presence and absence of RNAi, further studies were carried out with the GFP *C. elegans* strain PD4251. This strain produces GFP in all vulval and body wall muscles and was used to allow visualisation of any changes in the development of the vulva. This strain was also used to determine if the *protruding vulva* and *bag of worms* phenotypes were connected as nematodes that perished from *bag of worms* were often seen with protruding vulvas in early adulthood. The strain PD4251 (ccls4251 l; dpy-20 (e1282) IV) was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Centre for Research Resources (NCRR). PD4251 produces GFP in all body wall muscles and vulval muscles, with a combination of mitochondrial and nuclear localisation. The GFP nematodes were placed on agar plates containing 0μM and 500μM $CuSO_4$ and fed bacteria expressing the pPD129.36 control or the RNAi construct. Nematodes were left to develop and photographs were taken under UV light. ***Figure 7.5*** shows sample photographs of the PD4251 strain exposed to 0μM and 500μM $CuSO_4$ with RNAi of Ctr and CutC.

The photographs in ***Figure 7.5*** shows abnormal vulval development in nematodes exposed to RNAi of Ctr and CutC. In control nematodes on 0μM $CuSO_4$ a clear vulval valve can be seen allowing the normal passage of eggs. Whilst at 500μM *$CuSO_4$ bag of worms* develops.

With RNAi of Ctr at 500μM $CuSO_4$, abnormally protruding vulvas were seen alongside the early stages of *bag of worms*. In nematodes exposed to RNAi of CutC, vulvas that protrudes abnormally were observed at 0μM $CuSO_4$ and at 500μM $CuSO_4$ the *bag of worms* phenotype was clearly visible along with the *protruding vulva* phenotype.

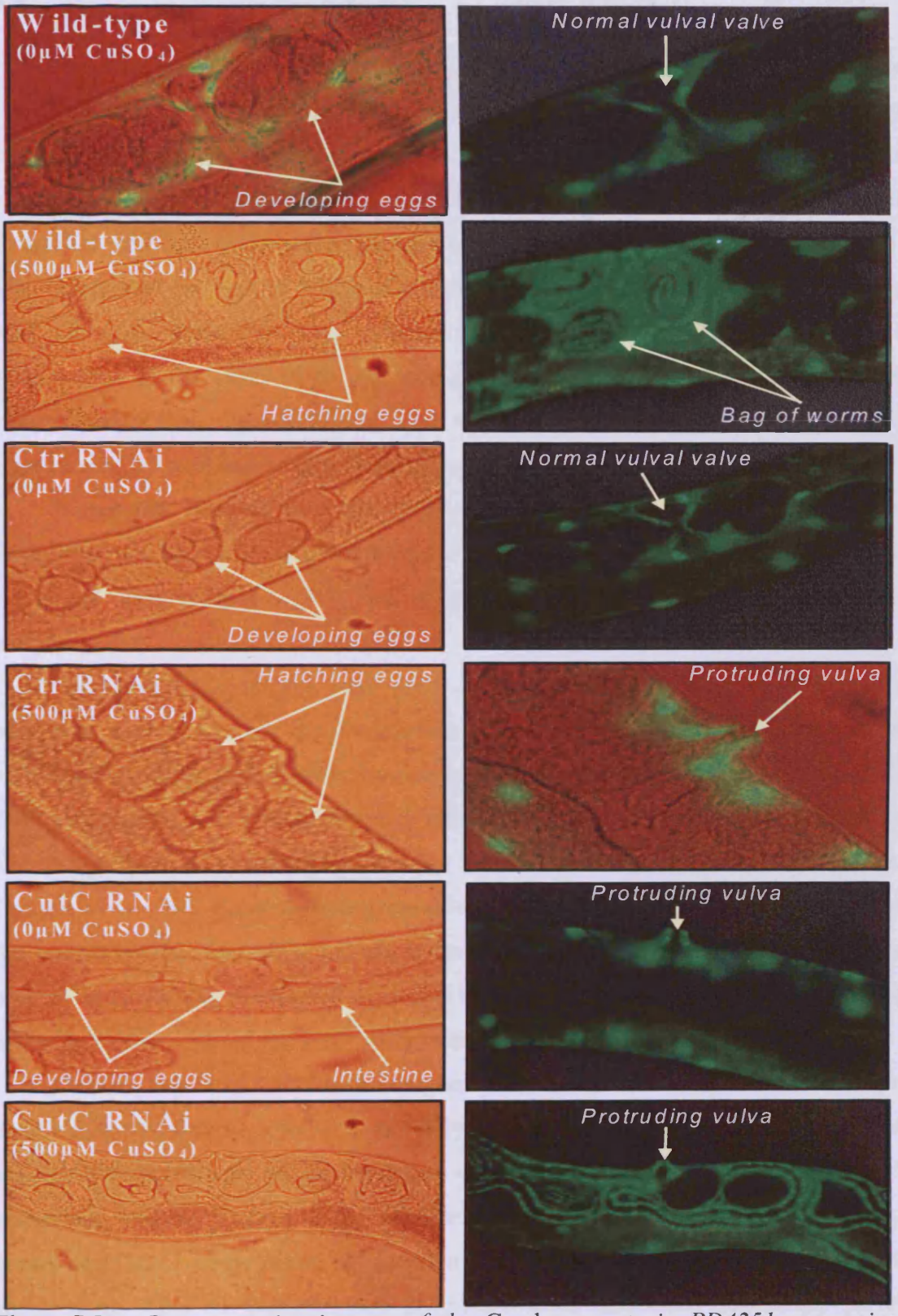

Figure 7.5 *Representative images of the* C. elegans *strain PD4251 expressing GFP in vulval and body wall muscles, with and without RNAi of Ctr and CutC on 0µM and 500µM $CuSO_4$.* C. elegans *visualised under white light in the left panel and with UV light on the right panel.*

7.3 Discussion

The various phenotypes seen in *C. elegans* on $CuSO_4$ plates are generally consistent with defects in the development of the vulva. Interestingly whereas *bag of worms* is seen in 70% of wild-type nematodes at 500μM $CuSO_4$, this phenotype is present with RNAi of the Ctr gene at all $CuSO_4$ concentrations with the percentage of nematodes with the abnormality increasing as the $CuSO_4$ concentration increased. Also nematodes exposed to RNAi of Ctr are smaller and have a slower growth rate than wild-type nematodes. This indicates that Ctr may have a direct role in the development of the vulva or in egg laying. In previous studies, data inferred from mutant phenotypes, has indicated that Ctr has a role in gametogenesis and reproduction, as well as a positive regulation on the body size and growth rate.

Nematodes exposed to RNAi of CutC were significantly smaller and had a reduced growth rate than control nematodes on all plates containing $CuSO_4$, but grew bigger and faster at 0μM $CuSO_4$, indicating that CutC is required in an environment containing Cu.

The various phenotypes seen in *C. elegans* on $CuSO_4$ plates were generally consistent with defects in the development of the vulva. Interestingly, *protruding vulvas* and *moulting defects* were seen in nematodes exposed to RNAi of CutC at all $CuSO_4$ concentrations. Thus nematodes with RNAi of CutC have a higher propensity to develop abnormally and CutC may have some involvement in vulval development. The use of the GFP strain showed *protruding vulva* and *bag of worms* phenotypes in the same animals, indicating that the abnormalities may be linked. The abnormally protruding vulva may not be allowing eggs to pass through and be laid, leading to the offspring hatching inside the adult and causing *bag of worms*.

Overall, RNAi of CutC in *C. elegans* did not show conclusive results in the demographic data as to whether it is likely to be directly involved in Cu import or export. The knockdown performed better compared to control sometimes with and sometimes without $CuSO_4$. This indicates that CutC has a complex role in Cu homeostasis in *C. elegans* and may have an intermediate function such as cytosolic chaperoning. Also the bioinformatic analysis showed that CutC has an α/β barrel structure which is usually indicative of enzymatic function.

The operon that CutC is found in is also very interesting in that there is an ubiquitin and aminopeptidase located either side of CutC. Due to the function and co-

regulation of operons it is likely that CutC performs a similar role to its two neighbouring genes. Unfortunately the diverse function of ubiquitin and aminopeptidase make it difficult to deduce a precise role of this operon.

The selective ubiquitin-mediated degradation of proteins is involved in the stress response, cell cycle, growth and differentiation, antigen processing, signal transduction, transcriptional regulation, DNA repair and apoptosis. Ubiquitination can also be used to modify a protein's activity, to change its subcellular location, or to alter protein-protein interactions (Hall *et al.*, 2005). Aminopeptidases regulate the N-terminal modification of proteins and peptides for maturation, activation or degradation, and thereby also relate to a variety of biological processes (Sato, 2004).

As genes in operons are co-transcribed in *C. elegans* it suggets that CutC may be functionally related to ubiquitin and aminopeptidase. Therefore CutC could be involved in protein regulation and turnover, possibly as an enzyme in the regulation of proteins involved in Cu homeostasis such as the stress response to elevated levels of Cu in the environment or as a regulator of Cu importers.

Chapter 8

General Discussion

Chapter 8

General Discussion

8.1 General Discussion

Increases in global industrialisation are causing a growing release of pollution into the environment. This environmental contamination of soil has become a threat to the continued existence of many plant and animal communities in our ecosystem and will eventually have an impact on Mankind. Pollution, particularly by heavy metals, is a significant problem as they are non-biodegradable and thus able to accumulate in ecological systems. The heavy metal Cu though toxic in excess is also an essential trace element that serves as a cofactor in many critical biological processes such as respiration, iron transport and oxidative stress protection.

It is important to assess the effects of heavy metal pollution on the ecosystem and their natural communities as environmental pollution impacts on an organism's genomic utilisation and thus results in consequences for its biology, ultimately affecting population dynamics. Therefore the overall objective of this project was to investigate the whole organism response to Cu toxicity in the soil dwelling nematode, *C. elegans* by analysing changes in life cycle traits; and to determine the expression profile and functional significance of two putative genes involved in Cu metabolism in *C. elegans* using QPCR and RNAi.

To facilitate this objective the first aim was to perform toxicity tests in *C. elegans* to determine the whole organism response to Cu. Preliminary investigations were necessary to determine if $CuSO_4$ should also be added to the bacterial food source on the plates as well in the NGM agar without significantly affecting the actual dose of $CuSO_4$ that the nematodes received. Results from Atomic Absorption spectrophotometry (AAS) confirmed that $CuSO_4$ could be added to the bacteria before spreading onto the agar, to ensure that the nematodes were fully exposed to the Cu, without resulting in any significant increase in the amount of Cu present (***Figure 3.1***).

Previous studies have shown that Cu and Fe homeostasis are intertwined (De

De Freitas *et al.*, 2003), thus the concentration of Fe in the NGM agar and its EC50 in *C. elegans* was also determined. The 3 day EC50 of $FeSO_4$ in *C. elegans* was found to be 2mM, whilst the basal level of Fe present in the NGM agar was only 0.07mM. Therefore the level of Fe present in the agar was not as a sufficient concentration to affect the Cu toxicity experiments. The combined EC50 experiment for Cu and Fe (***Figure 3.5)*** showed that $CuSO_4$ is more toxic than $FeSO_4$ to *C. elegans* and the combination of both metals increases the detrimental effect on the nematodes in a synergistic manner. Which concurs with studies showing that Cu and Fe are co-transported due to the dependence of Cu as a cofactor for ferroxidase Fet3p (***Figure 3.2***). In addition Shi, *et al.*, 2003 reported that Fet3p may function in Cu detoxification by binding free Cu, thus suppressing the pro-oxidant activity that this metal ion exhibits in the generation of oxygen free radicals.

These initial experiments were carried out to determine the EC50 and LC50 of $CuSO_4$ in *C. elegans* in order for further studies to be performed at appropriate $CuSO_4$ concentrations. The 3 day EC50 of $CuSO_4$ were found to be 0.1mM. As the minimum inhibitory concentration (MIC) of *E. coli* on agar medium is 1mM (Spain and Alm, 2003), LC50 experiments were carried out in liquid culture to ensure the bacterial food source was not limiting. The 24 hour and 72 hour LC50 of $CuSO_4$ in C. *elegans* was determined to be 1.59mM and 0.29mM respectively. This concurs with a previous study where the 24 hour LC50 of $CuSO_4$ in *C. elegans* was predicted from ion characteristics to be 1.57mM (Tatara et al., 1998).

Interestingly the natural concentration of Cu in soil is as high as approximately 0.79mM in non-contaminated soil and 213mM in polluted soil Barceloux, 1999 and Fernandez *et al.*, 2005). Toxicity though not only depends on metal concentrations but also on the solubility, mobility and chemical speciation (Bell et al., 1991; Lorenz et al., 1997). All of these factors can influence bioavailability of metals, which will differ among taxonomic groups and will depend on the exposure route. For example, Castano et al, 1995 reported that the EC50 of earthworms is 24 to 63mM Cu. Problems of contaminated soils are currently an important issue that can potentially affect terrestrial and aquatic communities, owing to the drainage and surface runoff of toxic substances in water from contaminated sites Fernandez *et al.*, 2005).

At toxic concentrations of $CuSO_4$ (100μM and above), detrimental effects were seen on the *C. elegans* life cycle with overall a reduction in brood size and life span and an increase in generation time and brood period. Also developmental problems were observed with nematodes at toxic $CuSO_4$ concentrations exhibiting a smaller body size, reduced growth rate and development along with various phenotypic abnormalities. The results of whole organism Cu toxicity in *C. elegans* were concurrent with a study on the effects of Cu on the springtail *Proisotoma minutia* where Cu also caused a significant reduction in size, a decrease in growth rate and slower movement (Nursita *et al.*, 2005). This shows how elevated levels of Cu pollution in the environment can have detrimental consequences on an organism's life cycle and thus affect the population dynamics of the organism.

In this study low concentrations of $CuSO_4$ were typically beneficial to the nematodes while higher concentrations had detrimental consequences (***Figure 3.3*** and ***Figure 3.4***). This phenomenon is known as the hormesis effect, which is characterised by a low dose stimulation and high dose inhibition. This highlights the essential yet toxic nature of Cu. A deficiency in Cu compromises cellular antioxidant defences via a decreased capability to produce the enzyme super-oxide dismutase (SOD), thereby increasing the susceptibility to oxidative DNA damage (Gaetke and Chow, 2003). Paradoxically, though Cu is required for many biological processes, in excess of cellular needs Cu ions are highly toxic, causing cellular damage through oxidising proteins and lipids (Sambongi *et al.*, 1998). Cu possesses this potential toxicity to cells because of its ability to reversibly donate and receive electrons. Studies have shown that Cu can be responsible for the intracellular generation of superoxide and other reactive oxygen species (Kimura and Nishioka, 1997). When allowed to engage in uncontrolled redox chemistry in a cell, Cu can cause devastating and irreparable damage to proteins, lipids and DNA (Rees and Thiele, 2004) leading to cell death and aging.

A lethal suppressor screen was performed in order to isolate and characterise Cu resistant mutants. Methanesulfonic acid ethyl ester (EMS) was utilised to chemically mutate the *C. elegans* DNA to induce Cu resistance. This was to determine if adaptive responses exist at the genetic level and what the effects of that are on the whole organism. The experiment resulted in 14 viable mutants which displayed varying degrees of resistance to Cu. 9 of which produced significantly more offspring

at 500μM $CuSO_4$ than the N2 controls. In addition it is worth noting that 7 of the EMS mutated strains showed no significant reduction in the number of progeny produced at 500μM $CuSO_4$ compared to 0μM $CuSO_4$ (***Table 3.6***). This indicates that these strains display a resistance to Cu toxicity, at least in terms of the brood size.

Interestingly, all Cu resistant mutants produced significantly less progeny than the wild-type strain on control plates. This observation correlates well with possible mutations in Cu importers, which could result in an impaired ability to import Cu into the cell. The detrimental consequences where there is a limited availability of Cu would be due to the inability to import sufficient Cu resulting in a deficiency. It is thought that Cu homeostasis is tightly controlled by the interactions of interconnected, but also independent components and our results concur with this. All mutants were resistant to the Cu toxicity in different ways (compared with the wild-type), suggesting the presence of differential genetics underlying the observed Cu tolerances.

The second main objective of the study was to investigate genes involved in Cu homeostasis in *C. elegans*. The putative Cu importer Ctr, was selected for investigation and has been previously well characterised as a plasma membrane bound Cu importer present in a wide range of organisms from bacteria to mammals. Ctr is a protein from a widely conserved family of high-affinity Cu transport proteins thought to mediates Cu uptake at the plasma membrane. As Cu homeostasis is a complex process controlled by many different proteins it was necessary to examine more than a single gene. Therefore a putative cytosolic chaperone encoding gene (CutC) implicated in Cu efflux from the cell, was also selected for further evaluation to contrast and compare aspects of Cu metabolism. CutC orthologues have been identified in many organisms including humans but yet remains uncharacterised.

Bioinformatic analysis confirmed that the *C. elegans* Ctr is a putative membrane-bound Cu transporter containing the Cu binding motif, MXXXM. Recent studies have revealed that the MXXXM motif in the second transmembrane domain plays a vital role in the chemistry of Cu sensing and uptake (Aller *et al.*, 2004). The Ctr protein also has the highly conserved GG4 motif which has an important role in the structure-function of Ctr transporters (Aller *et al.*, 2004)

Bioinformatic analysis also confirmed CutC as a putative cytosolic Cu chaperone in *C. elegans*. It possesses a highly conserved VTFHRA motif in the

hydrophobic core of the protein indicating an important role and histidine residues positioned for Cu binding either side of the protein pore. Interestingly the overall structure of the *C. elegans* CutC protein closely resembles an α/β barrel which is often found in enzymes (Reardon and Farber, 1995).

Reverse genetics were utilised in order to examine the expression pattern of Ctr and CutC and so investigate their role in Cu homeostasis. For expression analysis QPCR was utilised to determine the transcription levels of Ctr and CutC over different $CuSO_4$ concentrations. Gene expression for both Ctr and CutC was significantly down-regulated with increasing $CuSO_4$ concentrations. These results confirmed that the putative Ctr in *C. elegans* may have role as a Cu importer. At low Cu concentrations Ctr actively imports the essential heavy metal into the cell, but is transcription is down regulated in an environment containing high levels of Cu, to prevent over accumulation and intracellular toxicity. In addition to this a previous study on posttranslational regulation of Cu in yeast showed that the Ctr protein was found to be stable in Cu limited cells, but upon Cu treatment the protein was rapidly degraded (Eide, 1998).

These results were however unexpected for CutC, if it was as postulated a chaperone or exporter involved in Cu efflux. Though CutC may be a high affinity chaperone, working under basal non-toxic conditions and a different low affinity protein takes over when toxic levels of Cu are present in the environment.

The life cycle demographics of *C. elegans* were fully characterised over different Cu concentrations with and without RNAi of the putative Cu transporters. RNAi was exploited to knockdown Ctr and CutC in *C. elegans* and thereby determine the functional significance of the genes by fully characterising life cycle parameters in *C. elegans* upon exposure to $CuSO_4$.

Before performing the RNAi experiments, QPCR was used to validate the knock-down effect of RNAi. The expression of Ctr and CutC was significantly less in *C. elegans* exposed to RNAi (***Figure 5.14***). Thus with RNAi the use of exogenous Ctr or CutC interfered with the function of the endogenous target gene resulting in sequence specific silencing.

In all experiments $CuSO_4$ had significant detrimental consequences on the demographics of *C. elegans* with and without RNAi of Ctr and CutC. Overall the general toxic effect of Cu on *C. elegans* was a reduction in brood size and life span

and an increase in the brood period and generation time. Elevated levels of $CuSO_4$ had a significant effect on reducing the life span of *C. elegans* with both the control and RNAi tests. This is probably due to the fact that excess intracellular Cu causes the production of free radicals which leads to oxidative damage causing aging and death. Similarly high Cu concentrations reduced progeny in control and RNAi tests in a temporal manner, highlighting an essential role of Cu in development and embyogenesis.

Overall the results indicated that the putative Ctr gene has both a Cu dependant and Cu independent role in the production of progeny. At 0μM $CuSO_4$, nematodes with RNAi of Ctr produced significantly less progeny than the control, whilst on all plates containing $CuSO_4$, more progeny were produced in comparison. Cu is an essential metal required for embryogenesis and development, thus RNAi of the Ctr gene may have resulted in insufficient basal Cu uptake at 0μM $CuSO_4$, causing a reduction in brood size. It has been suggested that lysyl oxidase a Cu requiring enzyme might be important for formation of embryonic structures (Kuo *et al.*, 2001). Also cytochrome oxidase is a Cu dependant enzyme that is essential during embryogenesis, when cell proliferation and respiration is very active (Taperio *et al.*, 2003).

If the reduction in brood size was due to Cu deficiency, it is possible to suggest that this Ctr is the main transporter responsible for high affinity Cu uptake in *C. elegans*, at least in early embryogenesis; or that other Cu uptake genes in *C. elegans* are unable to fully compensate for the loss of this Ctr by RNAi. Though in yeast there is a redundancy in its Cu uptake genes. In addition to Ctr1, *S. cerevisiae* has a second high affinity Cu transporter gene, Ctr3, and a low affinity transporter gene, Ctr2 (Andrews, 2001). In *C. elegans* there are two Ctr-related homologues located close to this one in the genome, but their function remains uncertain.

In addition recent studies with Ctr1 KO mice have highlighted an essential Cu-independent function of Ctr in mammalian embryonic development since homozygous mutants died *in utero* (Sharp, 2003). Embryos were highly abnormal with severe growth retardation and widespread abnormalities attributable to Cu insufficiency (Kuo *et al*, 2001 and Lee *et al*, 2001).

Effects of RNAi of Ctr in nematodes are seen at 0μM $CuSO_4$, indicating that Ctr may have a role in high affinity Cu uptake. A low affinity importer would function

under conditions where Cu concentrations were sufficient. Whilst a high affinity transporter would be expressed when extracellular Cu concentrations were limited and active import was required. Therefore Ctr is likely to be a high affinity transporter as RNAi effects were observed at basal $CuSO_4$ concentrations.

The nematodes exposed to RNAi of Ctr had a higher EC50 value compared to control nematodes thus they are less affected by exposure to $CuSO_4$ in terms of number of progeny produced. This further validates the role of the putative Ctr as a Cu importer. Also with nematodes exposed to Ctr RNAi, the percentage survival did not actually decrease with increasing $CuSO_4$ concentrations. This is consistent with the hypothesis that Ctr functions as a Cu importer with a role in high affinity Cu uptake as RNAi effectively reduced the amount of Cu imported into the cell allowing the nematode to survive at higher Cu concentrations.

Nematodes exposed to RNAi of CutC performed better at lower $CuSO_4$ concentrations but less well at higher concentrations. They produced significantly more offspring than control at 20 and 100μM, but 30% less offspring at 500μM $CuSO_4$. This indicates that CutC may be involved in the efflux of Cu from the cell or in intracellular storage of free Cu ions, as when the function of the gene is disrupted by RNAi the nematodes are less able to tolerate elevated Cu concentrations. Previous studies have shown that in *E. coli* the CutC mutant is Cu sensitive and accumulates Cu but has apparently normal kinetics of Cu uptake. Thus the CutC gene was postulated to encode for an efflux protein which removes excess Cu from the cytoplasm (Gupta *et al.*, 1995).

The $CuSO_4$ EC50 with RNAi of CutC was higher compared to control nematodes, but at concentrations above a threshold of 293μM $CuSO_4$, Cu proved to be more toxic to the RNAi exposed nematodes than to controls, indicating that CutC may have a complex role in the efflux of Cu from the cell or in intracellular storage of free Cu ions. Nematodes exposed to RNAi of CutC had lower LC50 values than the controls and were less able to survive at elevated concentrations of $CuSO_4$, further indicating at a possible role for CutC in Cu efflux or intracellular storage. From previous studies in other organisms it was postulated that CutC encodes for an efflux protein which removes excess Cu from the cytoplasm (Gupta *et al.*, 1995).

The generation time was significantly increased at 100μM and 500μM $CuSO_4$, in both the control and RNAi exposed nematodes, indicating that toxic levels of Cu

has a detrimental effect on development in *C. elegans*. Though nematodes exposed to the RNAi of Ctr had a significantly shorter generation time than control nematodes at 500μM $CuSO_4$. This may be because the function of the Cu transporter is disrupted, so less Cu is imported into the cell allowing the nematode to perform better at higher Cu concentrations. The brood period also generally increased with rising concentrations of $CuSO_4$, and was significantly longer at 500μM $CuSO_4$ in both control and RNAi exposed nematodes. Elevated levels of $CuSO_4$ had a significant detrimental effect on the life span of *C. elegans* with both the control and RNAi tests. This is probably due to the fact that excess intracellular Cu causes the production of free radicals which leads to oxidative damage causing aging and death (***Section 1.12***).

Nematodes exposed to both RNAi of Ctr and CutC were significantly smaller and had a slower growth rate than control nematodes on all plates containing $CuSO_4$, but grew bigger and faster at 0μM $CuSO_4$. Interestingly the *bag of worms* phenotype was present with RNAi of the Ctr at all $CuSO_4$ concentrations with the percentage of nematodes with the abnormality increasing with rising $CuSO_4$ concentrations. This indicates that Ctr may have a direct role in the development of the vulva or in egg laying. In previous studies, data inferred from mutant phenotypes, has indicated that Ctr has a role in gametogenesis and reproduction, as well as a positive regulation on the body size and growth rate (WormBase web site, http://www.wormbase.org, release, date 2005).

The phenotypes *protruding vulvas* and *moulting defects* were seen in nematodes exposed to RNAi of CutC at all $CuSO_4$ concentrations. Thus nematodes with RNAi of CutC have a higher propensity to develop abnormally and CutC may have some involvement in vulval development.

The use of the GFP strain showed *protruding vulva* and *bag of worms* phenotypes in the same animals, indicating that these abnormalities may be linked. The abnormally protruding vulva may not be allowing eggs to pass through and be laid, leading to the offspring hatching inside the adult and causing *bag of worms*.

Overall, QPCR and RNAi of the CutC and Ctr genes resulted in different demographic and phenotypic effects, further indicating at their different and complex roles in Cu metabolism. RNAi of Ctr in *C. elegans* would indicate it to function as a high affinity importer of Cu. Whilst RNAi of CutC in *C. elegans* did not show conclusive results as to whether it is likely to be simply involved in Cu efflux or

storage. Its complex role in Cu homeostasis in *C. elegans* may have an intermediate function such as cytosolic chaperoning. Bioinformatics analysis indicates that CutC is likely to be involved in protein regulation and turnover, possibly as an enzyme in the regulation of proteins involved in Cu homeostasis such as the stress response to elevated levels of Cu in the environment or as a regulator of Cu proteins.

Interestingly CutC is located in the centre of an operon with an ubiquitin regulatory protein upstream and a leucine aminopeptidase downstream. The upstream ubiquitin regulatory protein UBXD2 inactivates proteins by the attachment of ubiquitin. This process called ubiquitination acts as a tag by which protein-transport machinery ferries the protein to the proteasome for degradation. The ubiquitination pathway degrades a multitude of cellular proteins, including cell cycle regulators, growth- and differentiation-controlling factors, transcriptional activators, cell-surface receptors and ion channels, endoplasmic reticulum proteins, antigenic proteins and abnormal and misfolded proteins (Hall. *et al.*, 2005). Ubiquitinylation can also be used to modify a protein's activity, to change its subcellular location, or to alter protein-protein interactions. The gene downstream of CutC, lap-1, encodes a homolog of the zinc metalloprotease aminopeptidase. Leucine aminopeptidases are exopeptidases which are presumably involved in the processing and regular turnover of intracellular proteins and are implicated in the final steps of intracellular protelolysis (Bartling and Weiler, 1992). (Saric *et al.*, 2004).

It has been suggested that the final steps in ubiquitin-proteasome pathway involves aminopeptidases, which very rapidly digest the small proteasome products (Saric *et al.*, 2004). Therefore the ubiquitin regulatory protein and leucine aminopeptidase found either side of CutC in the operon are likely to be co-expressed as it is probable that they work together in protein regulation. As operons usually co-regulate genes that make proteins with related functions (Blumenthal and Gleason, 2003) it is reasonable to suggest that CutC also has a role in protein regulation in Cu homeostasis.

In summary, the whole organism response to Cu toxicity response has been determined in *C. elegans*. Cu tolerant mutants were created and phenotypic effects examined in order to assess adaptive responses to heavy metal exposure. Genes have also been positively identified as having roles in Cu homeostasis, Ctr as a high affinity Cu importer and CutC possibly with a complex role in regulation.

Our understanding of Cu homeostasis and heavy metal toxicity can be greatly facilitated by the use of model organisms such as *C. elegans*. These models organisms provide us with an invaluable tool in determining the genes involved and their function in Cu metabolism and serve to support our efforts at understanding Cu homeostasis in higher organisms.

8.2 Future work

To further evaluate the role of Ctr and CutC in *C. elegans,* expression patterns of each homologue could be determined using green fluorescent proteins (GFPs) coupled to gene promoters in the presence and absence of Cu. This approach could also aid in assessing the specificity of these genes in response to Cu versus other heavy metals.

Further insight into the function of Ctr and CutC could be gained by investigating the other genes in the operon. Multiple knockouts of Ctr homologues in the *C. elegans* genome could be created to further assess the role of the putative Cu transporter. Additionally functional complementation studies could be performed in yeast, to confirm that the putative Ctr does sequester Cu.

It would be interesting to identify the mutated genes responsible for conferring Cu resistance in the *C. elegans* generated from the lethal suppressor screen. This could be facilitated by the use of Amplified fragment length polymorphism (AFLP) PCR, a highly sensitive tool used to detect DNA polymorphisms. On a genome wide-scale microarray technology could be utilised to identify Cu responsive genes in *C. elegans.*

9

References

9 References

Alexander, E., Pharm, D. and Steek, T. R. (1999). **The viable-but-nonculturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum***. Appl. *Environ. Microbiol.*, (65): 3754-3756.

Andrews, N. C. (2001). **Mining copper transport genes**. *PNAS,* (98): 6543-6545.

Andrews, N. C. (2002). **Metal transporters and disease**. *Current opinion in Chemical Biology*, (6): 181-186.

Aller, S. G., Eng, E. T., De Feo, C. J. and Unger, V. M. (2004). **Eukaryotic CTR Copper Uptake Transporters Require Two Faces of the Third Transmembrane Domain for Helix Packing, Oligomerization, and Function.** *J. Biol. Chem.*, (279): 51.

Babu, M (accessed 2007). **TIM Barrel Analysis.** Center for Biotechnology, Anna University. http://www.mrc-lmb.cam.ac.uk/genomes/madanm/articles/timanal.html.

Barceloux, D. G. (1999). **Copper**. *J. Toxicol Clin. Toxicol.*, (37): 217-30.

Bartling, D. and Weiler, E. W. (1992). **Leucine aminopeptidase from Arabidopsis thaliana. Molecular evidence for a phylogenetically conserved enzyme of protein turnover in higher plants** *Eur J Biochem.*, (1): 425-31.

Beers, J., Glerum, D. M. and Tzagoloff, A. (1997). **Purification, characterisation localisation of yeast Cox17p, a mitochondrial copper shuttle**. *J. Biol. Chem.*, (272): 33191-33196.

Bell, P. F., Chen, Y., Potts, W. E., Chaney, R. L. and Angle, J. S. (1991). **A reevaluation of the Fe(II), Ca(II), Zn(II), and proton formation constants of 4,7-diphenyl-1,10-phenanthrolinedisulfonate**. *Biol Trace Elem Res.*,(30): 125-44.

Bernstein, E., Denli, A. M. and Hannon, G. J. (2001). **The rest is silence.** *RNA,* (7): 1509-1521.

Beshgetoor, D. and Hambidge, M. (1998). **Clinical conditions altering copper metabolism in humans**. *Am. J. Clin. Nutr.,* (67)**:** 1017S-1021S.

Blumenthal, T., Evans, D., Link, C. D., Guffanti, A., Lawson, D., Thierry-Mieg, J., Thierry-Mieg, D., Chiu, W. L., Duke, K., Kiraly, M. and Kim, S. K. (2002). **A global analysis of *Caenorhabditis elegans* operons**. *Nature*, (417): 851-854.

Blumenthal, T. and Gleason, K. S. (2003). ***Caenorhabditis elegans* operons: form and function**. *Nat. Rev. Genet.,* (4):112-120.

Bolker, J. A. (1995). **Model systems in developmental biology**. *Bioessays*, (17): 451-455.

Bosher, J. M. and Labouesse, M. (2000). **RNA interference: genetic wand and genetic watchdog**. *Nat. Cell Biol.,* (2): e31-6.

Bremner, I. (1998). **Manifestations of copper excess**. *Am. J. Clin. Nutr.,* (67): 1069S–1073S.

Brown, N. L., Rouch, D. A. and Lee, B. T. (1992). **Copper resistance determinates in bacteria**. *Plasmid*, (27): 41-51.

Buettner, G. R. (1993). **The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate**. *Arch. Biochem. Biophys.,* (300): 535-543.

Castano, A., M., Vega, M. and Tarazona, J. V. (1995). **Acute toxicity of selected metals and phenols on RTG-2 and CHSE-214 fish cell lines.** *Bull Environ Contam Toxicol.*, (55): 222-229.

Cavet, J. S, Borrelly, G. P. M. and Robinson, N. J., 2003. **Zn, Cu and Co in cyanobacteria: selective control of metal availability**. *FEMS Microbiology Reviews*, (27): 165-181.

Cordano, A., Baertl, J. M. and Graham, G. G. (1964). **Copper deficiency in infancy.** *Pediatrics*, (34): 324-36.

Corpet, F. (1988). **Multiple sequence alignment with hierarchical clustering.** *Nucl. Acids Res.*, (16): 10881-10890.

Croll, N., A. and Matthews B. M. (1997). **Biology of nematodes.** Blackie and Son limited, London.

Cserzo, M., Wallin, E., Simon, I., von Heijne, G. and Elofsson, A. (1997). **Prediction of transmembrane alpha-helices in procariotic membrane proteins: the Dense Alignment Surface method**. *Prot. Eng. vol.*, (6): 673-676.

Culetto, E. and Sattelle, D. B. (2000). **A role of *Caenorhabditis elegans* in understanding the function and interactions of human disease genes**. *Human Molecular Genetics,* (9): 869-877.

Culotta, V. C., Komp, L. W. J., Strain, J., Casereno, R. L. B., Krems, B. and Gitlin, J. D. (1997). **The copper chaperone for superoxide dismutase**. *J. Biol. Chem.*, (272): 23469-23472.

Dancis, A., Haile, D., Yuan, D. S. and Klausner, R. D. (1994). **The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake**. *J Biol Chem.*, (269): 25660-25667.

De Freitas, J., Wintz, H., Kim, J. H., Poynton, H., Fox, T. and Vulpe, C. (2003). **Yeast, a model organism for iron and copper metabolism studies**. *Biometals*, (16): 185-197.

Eide, D. J. (1998). **The molecular biology of metal ion transport in *Saccharomyces Cerevsiae***. *Annu. Rev. Nutr.*, (18): 441-469.

Expasy Tools website. www.expasy.org, (2005).

Fernandez, M. D., Cagigal, E., Vega, M. M., Urzelai, A., Babin, M., Pro, J. and Tarazona, J. V. (2005). **Ecological risk assessment of contaminated soils through direct toxicity assessment.** *Ecotoxicology and Environmental Safety,* (2): 174-84.

Finney, L. A. and O'Halloran, T. V. (2003). **Transition Metal Speciation in the Cell: insights from the Chemistry of Metal Ion Receptors**. *Science*, (300): 931-936.

Fong, S. T., Camakaris, J. and Lee, B. T. O. (1995). **Molecular genetics of a chromosomal locus involved in copper tolerance in *Escherichia coli* K-12**. *Mol. Microbiol.*, (15): 1127–1137.

Freedman, J. H., Ciriolo, M. R. and Peisach, J. (1989). **The role of glutathione in copper metabolism and toxicity**. *J. Biol. Chem.*, (264): 5598-605.

Gaetke, L. M. and Chow, C. K. (2003). **Copper toxicity, oxidative stress, and antioxidant nutrients**. *Toxicology,* (189): 147-163.

Ganonet, F. and Lauquin, G. J. M. (1998). **The *Saccharomyces cerevisiae* LYS7 gene involved in oxidative stress protection.** *Eur. J. Biochem.*, (251): 716-723.

Glerum, D. M., Shtanko, A. and Tzagoloff, A. (1996). **Characterisation of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase.** *J. Biol. Chem.,* (271): 14504-14509.

Grishok, A., Tabara, H. and Mello, C. G. (2000). **Genetic requirements for inheritance of RNAi in *C. elegans*.** *Science,* (287): 2494-2497.

Guex, N. and Peitsch, M. C. (1997). **SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling**. *Electrophoresis*, **(18):** 2714–2723.

Gupta, S. D., Gan, K., Schmid, M. B. and Wu, H. C. (1993). **Characterization of a temperature-sensitive mutant of *Salmonella typhimurium* defective in apolipoprotein N-acyltransferase.** *J. Biol. Chem.*, (268): 16551-16556.

Gupta, S. D., Lee, B. T. O., Camakaris, J. and Wu, H. C. (1995). **Identification of *cutC* and *cutF* (*nlpE*) genes involved in copper tolerance in *Escherichia coli*.** *Journal of Bacteriology*, (15): 4207-4215.

Gupta, S. D., Wu, H. C. and Rick, P. D. (1997). **A *Salmonella typhimurium* genetic locus which confers copper tolerance on copper-sensitive mutants of *Escherichia coli*.** *Journal of Bacteriology,* (179): 4977-4984.

Hall, J. F., Ellis, M. J., Kigawa, T., Yabuki, T., Matsuda, T., Seki, E., Hasnain, S. S. and Yokoyama, S. (2005). **Towards the high-throughput expression of metalloproteins from the *Mycobacterium tuberculosis* genome**. *J. Synchrotron Radiat.*, (12): 4-7.

Hannon, J. G. (2002). **RNA interference**. *Nature,* (418): 244-251.

Harrison, M. D. and Dameron, C. T. (1999). **Molecular mechanisms of copper metabolism and the role of the Menkes disease protein.** *J. Biochem. Mol. Toxicology,* (13): 93-106.

Herbik, A., Bolling, C. and Buckhout, T. J. (2002). **The involvement of a multicopper oxidase in iron uptake by the green alage Chlamydomonas reinhardtii.** *Plant Physiol.*, (4): 2039-48.

Herman, R. K., Kari, C. K. and Hartman, P. S. (1982). **Dominant X-chromosome nondisjunction mutants of *Caenorhabditis elegans***. *Genetics*, (102): 379-400.

Hopkin, S. P. (1989). **Ecophysiology of metals in terrestrial invertebrates**. Elsevier Applied Science, London.

Ishmael, J., Gopinath, C. and Howell, J. M. (1972). **Experimental chronic copper toxicity in sheep – histological and histochemical changes during development of lesions in liver**. *Res. Vet. Sci.*, (12): 358-366.

Jacob, R. A., Skala, J. H., Omaye, S. T. and Turnlund, J. R. (1987). **Effect of varying ascorbic acid intakes on copper absorption ceruloplasmin levels of young men.** J. *Nutr.*, (12): 2109-15.

Järup (2003). **Hazards of heavy metal contamination**. *British Medical Bulletin*, (68): 167-182.

Kadiiska, M. B., Hanna, P. M. and Mason, R. P. (1993). ***In vivo* ESR spin trapping evidence for hydroxyl radical-mediated toxicity of paraquat and copper in rats**. *Toxicol. Appl. Pharmacol.*, (123): 187-192.

Kimura, T. and Nishioka, H. (1997). **Intracellular generation of superoxide by copper sulphate in *Escherichia coli***. *Mutat. Res.*, (389): 237-242.

Klein, W. J. Jr., Metz, E. N. and Price, A. R. (1972). **Acute copper intoxication. A hazard of hemodialysis.** *Arch. Intern. Med.*, (4): 578-82.

Klomp, A. E., Tops, B. B., Van Denberg, I. E., Berger, R. and Klomp, L. W. (2002). **Biochemical characterization and subcellular localization of human copper transporter 1 (hCTR1).** *Biochem J.*, (364): 497–505.

Knight, S. A., Labbe, S., Kwon, L. F., Kosman, D. J. and Thiele, D. J. (1996). **A widespread transposable element masks expression of a yeast copper transport gene.** *Genes Dev.*, (10): 1917-1929.

Knobeloch, L., Schubert, C., Hayes, J., Clark, J., Fitzgerald, C. and Fraundorf. (1998). **Gastrointestinal upsets and new copper plumbing – is there a connection?** *WMJ*, (1): 49-53.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001). **Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes.** *J Mol Biol.*, (3):567-80.

Kuo, Y. Zhou, M., B., Cosco, D. and Gitschier, J. (2001). **The copper transporter CTR1 provides an essential function in mammalian embryonic development.** *Proc Natl. Acad. Sci. U. S. A.*, (98): 6836-6841.

Labbe, S., Peña, M. M., Fernandes, A. R. and Thiele, D. J. (1999). **A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*.** *J Biol Chem.*, (274): 36252-36260.

Lee, J., Prohaska, J. R. and Thiele, D. J. (2001). **Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development.** *Proc. Natl. Acad. Sci. U. S. A.*, (98): 6842-6847.

Lee, J., Pena, M. M., Nose, Y. and Thiele D. J. (2002). **Biochemical characterization of the human copper transporter Ctr1.** *J. Biol. Chem.*, (277): 4380-7.

Leeper, G. W. (1978). **Pollution engineering and technology 6; Managing the heavy metals on the land**. Marcel Dekker, inc. New York and Basel.

Lenntech (2004) **Water treatment and air purification**, Holding B.V. http://www.lenntech.com/home.htm

Li, J., Ji, C., Chen, J., Yang, Z., Wang, Y., Fei, X., Zheng, M., Gu, X., Wen, G., Xie, Y. and Mao, Y. (2005). **Identification and charactreization of a novel Cut family cDNA that encodes human copper transporter protein CutC.** *Biochem. Biophys. Res. Commun.*, (337): 179-183.

Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V. and Culotta, V. C (1997). **A role for the *Saccharomyces cerevisiae* TAX1 gene in copper trafficking and iron transport.** *J. Biol. Chem.,* (272): 9215-9220.

Linder, M. C. (1991). **Biochemistry of copper.** New York, Plenum Press.

Llanos, R. M. and Mercer, J. F. (2002). **The molecular basis of copper homeostasis copper-related disorders.** *DNA Cell Biol.,* (21): 259-70.

Lorenz, S. E., Hamon, R. E., Holm, P. E., Domingues, H. C., Sequeria, E. M., Christensen, T. H. and McGrath, S. P. (1997). **Cadmium and zinc in plants and soil solutions from contaminated soils.** *Plant and Soil* (189): 21-31.

Lucas, L. C. and Lemons, J. E. (1992). **Biodegradtion of restorative metallic systems.** *Adv. Dent. Res.*, (6): 32-7.

Mattie, M. D., and Freedman J. H. (2003). **Copper-inducible transcription: Regulation by metal and oxidative stress-response pathways.** *Am J Physiol Cell Physiol.*, (22): C293-301.

McGuffin, L. J., Bryson, K. and Jones, D. T. (2000). **The PSIPRED protein structure prediction server.** *Bioinformatics*, (16): 404-405.

Montaser, A., Tetreault, C. and Linder, M. (1992). **Comparison of copper binding components in dog serum with those in other species.** *Proc Soc Exp Biol Med.*, (200): 321-329.

Moolenaar, S. W. and Lexmond, T. M. (1999). **Heavy Metal Balances, Part I. General Aspects of Cadmium, Copper, Zinc and Lead Balance Studies in Agro-Ecosystems.** *Journal of Industrial Ecology*, (2): 45-60.

Muller, J., Landgraf, F. and Trabert, W. (1998). **Schizophrenia-like symptoms in the Westphal-Strumpell variation of Wilson disease.** *Nervenarzt*, (69): 264-268.

Nagano, N., Hutchinson, E. G. and Thornton, J. M. (1999). **Barrel structures in proteins: automatic identification and classification including a sequence analysis of TIM barrels.** *Protein Sci.*, (10): 2072-2084.

Nasarabadi, S., Milanovich, F., Richards, J. and Belgrader, P. (1999). **Simultaneous detection of TaqMan probes containing Fam and Tamra reporter fluorophores.** *Biotechniques,* (27): 1116-1118.

Nieboer, E. and Richardson, D. H. S. (1980). **The replacement of the non-descript term "heavy metals" by a biologically and chemically significant classification of metal ions.** *Environ. Pollut.*, (1B): 3-26.

Nies, D. H. and Silver, S. (1995). **Ion efflux systems involved in bacterial metal resistances**. *J Ind Microbiol.*, (14): 186–199.

Notten, M. J. M , Oosthoek, A. J. P., Rozema, J. and Aerts, R. (2005). **Heavy metal concentrations in a soil-plant-snail food chain along a terrestrial soil pollution gradient.** *Environ Pollut.*, (1): 178-90.

Nriagu, J. O. (1979). **Copper in the environment; Part II: Health Effects**. John Wiley & Sons, New York.

Nriagu, J. O. and Pacyna, J. M. (1998). **Quantitative Assessment of Worldwide Contamination of Air, Water and Soils by Trace Metals**. *Nature,* (333): 134-139.

Nursita, A. I., Singh, B. and Lees, E. (2005). **The effects of cadmium, copper, lead, and zinc on the growth and reproduction of *Proisotoma minuta* Tullberg (Collembola)**. *Ecotoxicol Environ Saf.*, (60): 306-314.

Odermatt, A. and Solioz, M. (1995). **Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of *Enterococcus hirae***. *J Biol Chem.*, (270): 4349-4354.

Opazo, C., Barría, .M. I., Ruiz, F. H. and Inestrosa, N. C. (2003). **Copper reduction by copper-binding proteins and its relation to neurodegenerative diseases.** *Biometals*, (16): 91-98.

Outten, F. W., Huffman, D. L., Hale, J. A. and O'Halloran, T. V. (2001). **The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli.*** *J. Biol. Chem.*, (276): 30670-30677.

Peitsch, M. C. (1995). **Protein modelling by E-Mail.** *BioTechnology*, (13): 658–660.

Pena, M. M., Koch, K. A. and Thiele, D. J. (1998). **Dynamic regulation of copper uptake and detoxification genes in *Saccharomyces cerevisiae.*** *Mol. Cell. Biol.*, (18): 2514-2314.

Pena, M. M. O., Lee, J. and Thiele, D. J. (1999). **A delicate balance: homeostatic control of copper uptake and distribution.** *J Nutr.*, (129): 1251–1260.

Petris, M. J., Smith, K., Lee, J. and Thiele, D. J. (2003). **Copper-stimulated endocytosis and degradation of the human copper transporter, hCtr1.** *J. Biol. Chem.*, (11): 9639-46.

Portnoy, M. E., Schmidt, P. J., Rogers, R. S. and Culotta, V. C. (2001). **Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae.*** *Mol. Genet. Genomics,* (265): 873-882.

Powell, S. R. (2000). **The antioxidant properties of zinc.** *J. Nutr.*, (130): 1447S-1454S.

Prohaska, J.R., and Gybina, A.A. (2004). **Intracellular copper transport in mammals.** *J. Nutr.*, (134): 1003-1006.

Prusiner, S. B. (1995). **Prion diseases.** *Scientific American.*, (272): 48-56.

Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schimdt, P., Culotta, V. C., Penmner-Hehn, J. E. and O'Halloran, T. V. (1997). **Metal ion chaperone function of the soluble Cu(I) receptor Atx1.** *Science,* (278): 853-856.

Puig, S., Lee, J., Lau, M. and Thiele, D. J. (2002). **Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper transport.** *J. Biol. Chem.,* (29): 277.

Reardon, D., and Farber, G. K. (1995). **The structure and evolution of alpha/beta barrel proteins**. *Faseb J.,* (9): 497-503.

Rees, E. M. and Thiele, D. J. (2004). **From aging to virulence: forging connections through the study of copper homeostasis in eukaryotic microorganisms**. *Current Opinion in Microbiology*, (71): 1-10.

Rensing, C. and Grass, G. (2003). ***Escherichia coli* mechanisms of copper homeostasis in a changing environment**. *FEMS Microbiology Reviews,* (2-3): 197-213.

Riddle, D., Blumenthal, T., Meyer, B., Priess, J. (1997). ***C. elegans* II**. Cold Spring Harbor Laboratory Press. Fairview, N.Y.

Rogers, S. D., Bhave, M. R., Mercer, J. F. B., Camakaris, J. and Lee, B. T. O. (1991). **Cloning and characterisation of *cutE*, a gene involved in copper transport in *Escherichia coli.*** *Journal of Bacteriology*, 6742-6748.

Rouch, D., Camakaris, J. and Lee, B. T. O. (1989). **Copper transport in *E. coli***. p. 469-477. *In* D. H. Hamer, and D. R. Winge (ed.), *Metal ion homeostasis: molecular biology and chemistry*. Alan R. Liss, Inc., New York, N.Y.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988). **Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase**. *Science,* (239): 487-491.

Salomons, W. and Forstner, U. (1984). **Metals in the Hydrocycle**, New York, USA.

Sambongi, Y., Wakabayaahi, T., Yoshimizu, T. and Futai, M. (1998). **Physiological roles of *C. elegans* copper transport ATPase**. *European Worm Meeting abstract.*

Sambrook, J., Fitsch, E. F. and Maniatis, T. (Eds) (1989). **Molecular cloning - a laboratory manual**. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, USA.

Saric, T., Graef, C. I. and Goldberg, A. L. (2004). **Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases**. *J. Biol. Chem.*, (45): 46723-46732.

Satchell, J. E. (1983). **Earthworm Ecology, from Darwin to vermiculture**. Chapman and Hall Limited, London.

Sato, Y. (2004). **Role of aminopeptidase in angiogenesis**. *Biol. Pharm. Bull.*, (27): 772-776.

Sayle, R. A. and Milner-White, E. J. (1995). **RASMOL: biomolecular graphics for all.** *Trends Biochem Sci.*, (9):374.

Saylor, W. W. and Leach, R. M. (1980). **Intracellular distribution of copper and zinc in sheep-effect of age and dietary levels of the metals**. *J. Nutr.*, (110): 448-459.

Schwede, T., Kopp, J., Guex, N. and Peitsch, M. C. (2003). **SWISS-MODEL: an automated protein homology-modeling server.** *Nucleic Acids Research,* (13):3381-3385.

Scott, K. C. and Turnlund, J. R. (1994). **A compartmental model of zinc metabolism in adult men used to study effects of three levels of dietary copper**. *Am. J. Physiol.*, (267): E165-E173.

Semple, A. B., Parry, W. H. and Phillips, D. E. (1960). **Acute copper poisoning. An outbreak traced to contaminated water from a corroded geyser.** *Lancet.*, (2): 700-701.

Sharp, P. A., (2003). **Ctr1 and its role in body copper homeostasis.** *IJBCB,* (35): 288-291.

Shi, X., Stoj, C., Romeo, A., Kosman, D. J. and Zhu, Z. (2003). **Fre1p Cu2+ reduction and Fet3p Cu1+ oxidation modulate copper toxicity in *Saccharomyces cerevisiae*.** *J. Biol. Chem.,* (278): 50309-50315.

Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V., Kamath, R. S., Fraser, A. G., Ahringer, J. and Plasterk, R. H. (2003). **Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions.** *PLoS Biol.,* (1): e12.

Snyder, W. B., Davis, L. J., Danese, P. N. Cosma, C. L. and Silhavy, T. J. (1995). **Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway**. J. Bacteriol., (177): 4216-4223.

Spain, A. and Alm, E. (2003). **Implications of microbial heavy metal tolerance in the environment.** *Rev in undergraduate research,* (2): 1-6.

Spitalny, K. C., Brondum, J., Vogt, R. L., Sargeant, H. E. and Kappel, S. (1984). **Drinking-water-induced copper intoxication in a Vermont family**. *Pediatrics*, (6): 1103-6.

Strausak, D., Mercer, .J. F. B., Dieter, H. H., Stremmel, W. and Multhaup, G. (2001). **Copper disorder with neurological symptoms: Alzheimer's, Menkes, and Wilson diseases**. *Brain Research Bulletin*, (55): 175-185.

Swain, S. C, Keusekotten, K., Baumeister, R. and Stürzenbaum S. R. (2004). **C. elegans Metallothioneins: New Insights into the Phenotypic Effects of Cadmium Toxicosis**. . J. Mol. Biol., (341): 951-959.

Tapiero, H., Townsend, D. M. and Tew, K. D. (2003). **Trace elements in human physiology and pathology. Copper.** *Biomedicine and Pharmacotherapy*, (57): 386-398.

Tatara, C. P., Newman, M. C., McCloskey, J. T. and Williams, P. L. (1998). **Use of ion characteristics to predict relative toxicity of mono-, di-, and trivalent metal ions: *Caenorhabditis elegans* LC50**. *Aquatic toxicology*, (42): 255-269.

Townsend, D. M., Tew, K. D. and Tapiero, H. (2003). **The importance of glutathione in human disease**. *Biomed. Pharmacother.*, (57): 145-155.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itho, K., Hakashima, R., Yaono, R. and Yoshikawa, S. (1995). **Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A.** *Science,* (269): 1069-1074.

Turnlund, J. R., Scott, K. C., Peiffer, G. L., Jang, A. M., Keyes, W. R., Keen, C. L. and Sakanashi, T. M. (1997). **Copper status of young men consuming a low-copper diet.** *Am. J. Clin. Nutr.*, (65): 72-8.

Tusnády, G.,E. and Simon, I. (1998). **Principles governing amino acid composition of integral membrane proteins: application to topology prediction.** *J. Mol. Biol.*, (283): 489-506.

Tusnády, G.,E. and Simon, I. (2001) **The HMMTOP transmembrane topology prediction server.** *Bioinformatics,* (17): 849-850.

UNEP (United Nations Environmental Programme), (1994). **UNEP Data Report**. UNEP, Nairobi, Kenya.

Vallee, B. L. and Ulmer, D. D. (1972). **Biochemical effects of mercury, cadmium and lead.** *Ann. Rev. Biochem.*, (41): 91-128.

Van Geen. A and Luoma, S. N. (1999) **The impact of human activities on sediments of San Francisco Bay: An overview**, *Marine Chemistry*, (64): 1-6.

Vilaplana, J., Romaguera, C. Grimalt, F. and Cornellana, F. (1991). **New trends in the use of metals in jewellery.** *Contact Dermatitis*, (3): 145-148.

Vulpe, C. D. and Packman, S. (1995). **Cellular Copper transport.** *Annu. Rev. Nutr.*, (15): 293-322.

Waalkes, M. P. (1995). **Metal carcinogenesis**. Pages 47-70 in Metal Toxicity. Goyer, R.A., C.D. Klassen and M. P. Waalkes, eds. Academic Press, California.

Wickens, A. P. (2001). **Ageing and the free radical theory**. *Respiration Physiology,* (128): 379-391.

Winge, D. R. and Mehra, R. K. (1990). **Host defences against copper toxicity**. *Int. Rev. Exp. Pathol.*, (31): 47-83.

Wood, W. B. (1988). **The nematode *Caenorhabditis elegans.*** Cold spring harbour laboratory press, New York.

WormBase web site, http://www.wormbase.org, release WS130, (2005).

Zhu, Y. Q., Zhu, D. Y., Lu, H. X., Yang, N., Li, G. P. and Wang, D. C. (2005). **Purification and preliminary crystallographic studies of CutC, a novel copper homeostasis protein from Shigella flexneri.** *Protein Pept Lett.*, (8):823-6.

Zuckerman, B. M. (1980). **Nematodes as biological models, volume 1: behavioural and developmental models**. Academic press.

10

Appendix

10. Appendix

A.1 Extractable concentration of Cu determined by AAS

The concentration of Cu available was calculated from the nominal amount of $CuSO_4.7H_2O$ added in experiments using a scatter graph drawn from the AAS results (***Section 3.2***). The linear regression was determined and from the equation (y = 0.6314x + 6.9411) the nitric acid extractable concentration of Cu used throughout experiments was calculated (***Table A.1***).

Nominal amount of $CuSO_4.7H_2O$ (μM)	Extractable concentration of Cu determined by AAS (μM)
0	7
1	8
5	10
10	13
20	20
25	23
50	39
75	54
100	70
125	86
150	102
200	133
400	260
500	323
600	386
800	512
1000	638
2000	1270

Table A.1 *Concentrations of Cu used in experiments determined from the nominal amount of $CuSO_4.7H_2O$ added.*

A.2 DNA molecular weight markers

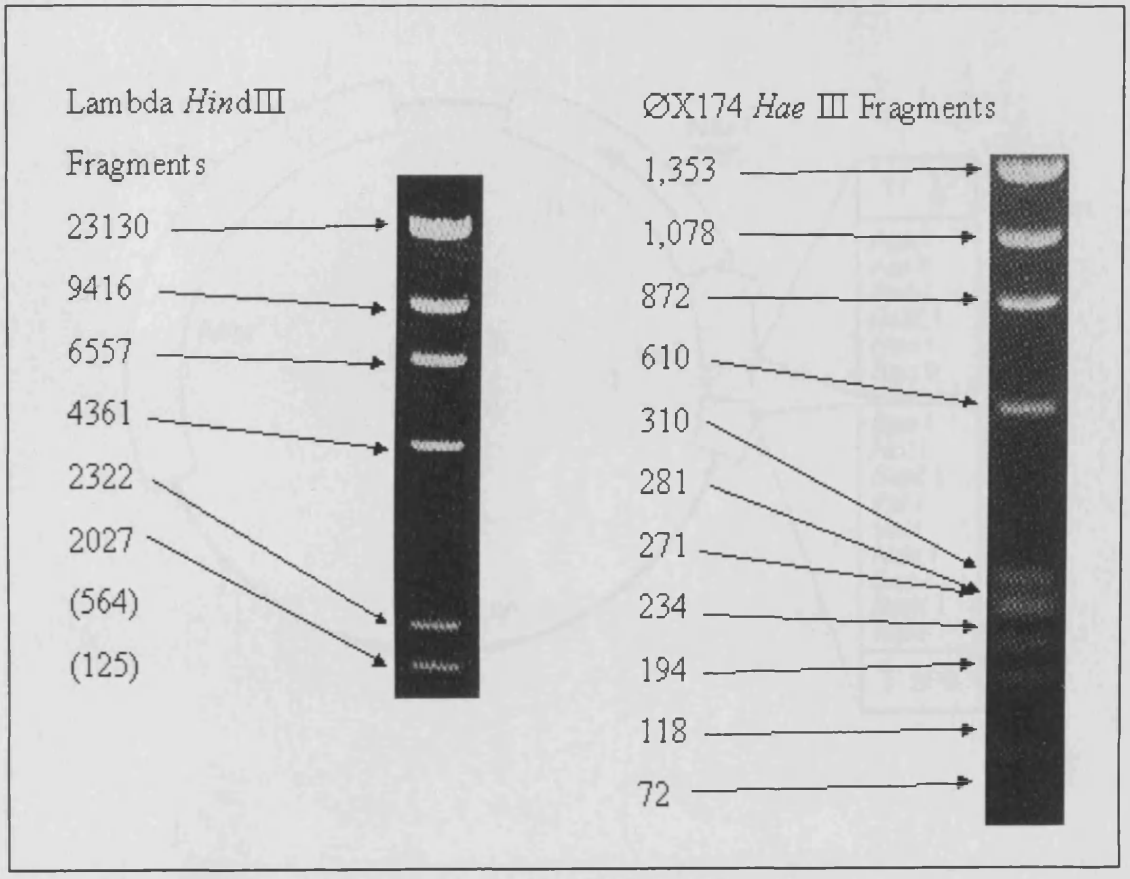

Figure A.1 *Molecular weights of DNA markers used in agarose gel electrophoresis. All values given in bp and gel photos adapted from Sigma catalogue, 2000.*

A.3 Vector maps

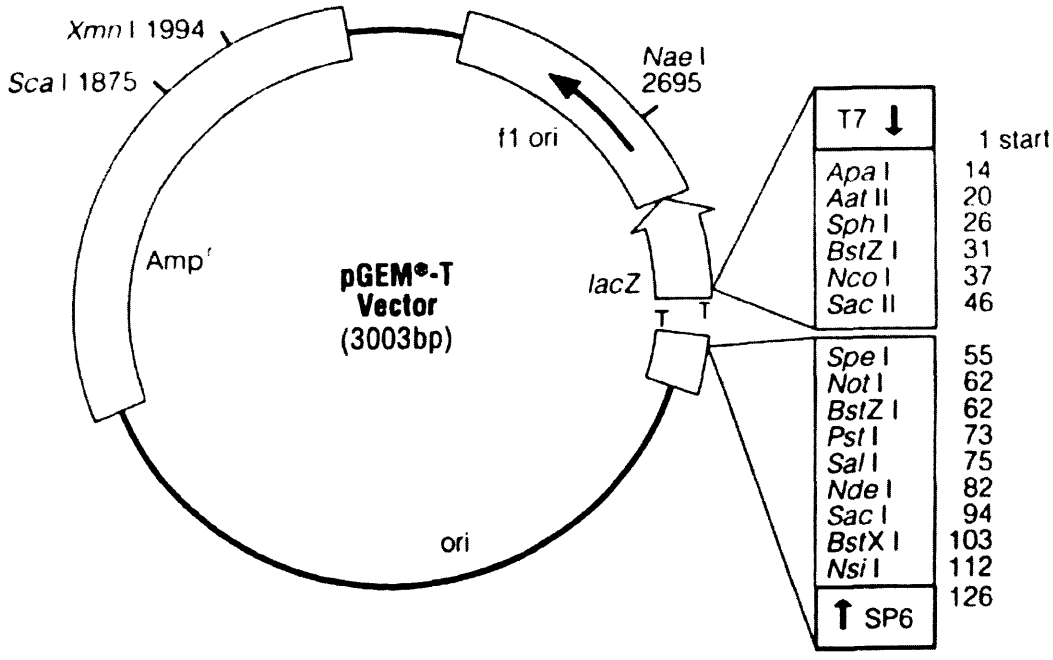

Figure A.2 *Plasmid map for pGEM-T, showing the cloning site and multiple restriction sites (reprinted from Promega catalogue, 2003).*

A.4 Standard PCR Primer sequences

Application	Primer	GC content	T_m (°C)	Sequence
Universal sequencing primers	M13 F	50% (18-mer)	53.7	5`-TGT AAA ACG ACG GCC AGT-3`
	M13 R	40% (20-mer)	53.2	5`-GGA TAA CAA TTT CAC ACA GG-3`
RNAi knockout in *C. elegans*	RNAi-CutC_F	45% (20-mer)	58.0	5`-GGAATTCC TCG ATA ATC TGG AAT CTG CG-3`
	RNAi-CutC_R	43% (21-mer)	60.0	5`-GGAATTCC GAT ACC AGT GAC ATT TAG TCC-3`
	RNAi-Ctrl_F	37% (19-mer)	52.0	5`-GGAATTCC ATG ATA CAA GGA CCT TTC A-3`
	RNAi-Ctrl_R	37% (19-mer)	52.0	5`-GGAATTCC GAG CAT AAG ACA ATA ACT G-3`
Gateway system (CACC adapters allows directional cloning into the Invitrogen entry vector)	CACC-CutC_F	42% (19-mer)	52.0	5`-CACC ATG ACA GAA ACA GAA TCC C-3`
	CACC-CutC_R	42% (19-mer)	52.0	5`-ATA ATT AAA CGG GGG CCA A-3`
	CACC-Ctrl_F (3)	26% (19-mer)	48.0	5`-CACC ATG AAT CAT AAT TCA ATG G-3`
	CACC-Ctrl_R (3)	26% (19-mer)	48.0	5`-ATA AAA ACT ATT CAC TCC T-3`
Bioinformatic work (primers to confirm annotation of the putative Ctrl gene in *C. elegans*)	CACC-Ctrl_F.2	42% (19-mer)	52.0	5`CACCATGCCGATTGC AGTTGTTA-3`
	CACC-Ctrl_R.2	42% (19-mer)	52.0	5`-CTA TAG CGA TGA TCC AGT T-3`
	CACC-Ctrl_R.3	42% (19-mer)	52.0	5`-AAC TAT TCA CTC CTT CCT C-3`
	CACC-Ctrl_F.7	42% (19-mer)	52.0	5`-CACC ATG ATG CAC ATG ATG ATG G-3`
	CACC-Ctrl_R.7	32% (19-mer)	50.0	5`-TTA TGA ACA ACA ACT TCC A-3`

Table A.2 *A List of primers and their details used for standard PCR.*

A.5 QPCR primer and probe sequences

Application	Primer/ Probe	GC content	T_m (°C)	Sequence	Size (bp)
Ctr QPCR	Forward primer	31%	58	5`-GGA AGC AAT TAA GTA TAA CAG AAG ACT AAT TC-3`	32
	Reverse primer	65%	58	5`-GGA GAG CAG GCG GGA AA-3`	17
	Ctr probe	35%	68	[6~FAM]AAA ACG ACA ATC ACC AAG CAA AAA AGA AAG CTA C[TAMRA~6~FAM]	34
CutC QPCR	Forward primer	38%	59	5`-TGA TTG ACA TGA TGT ACG ATC CAA-3`	24
	Reverse primer	50%	60	5`-TGG TGC CAC AGG ATT CGT TT-3`	20
	CutC probe	48%	70	[6~FAM]AAC CGG CTG ATG TTA ATG CTC CA[TAMRA~6~FAM]	23
rpa-1 QPCR	Forward primer	52%	59	5`-CCG GAA GAG ACA GAA GTG ATG AG-3`	23
	Reverse primer	65%	59	5`-GCC GCC AAC GTC GAG TT-3`	17
	Rpa-1 probe	48%	69	[6~FAM]TTC TTC ACA TCA ACT CCC TCG AGA GCC TT[TAMRA~6~FAM]	29

Table A.3 *A List of primer and probe sequences used for QPCR.*