Exploiting *C. elegans* to investigate the key combinatorial toxicology associated with the marine environment in the proximity of Jeddah city in the Red Sea

BY

Yaser Mohammed Sahl

Thesis submitted to Cardiff University for the degree of Doctor of Philosophy

December 2014

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

Signed ……..…… (candidate) Date ……

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD.

Signed ……..…… (candidate) Date ……

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references. The views expressed are my own.

Signed ……..…… (candidate) Date ……

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available online in the University's Open Access repository and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed ……..…… (candidate) Date ……

ACKNOWLEDGMENTS

There are several people to whom I remain indebted to for their support, inspiration and advice during my time as a PhD student. I would like to thank all laboratory members in Saudi Geological Survey, Jeddah, Saudi Arabia for their help with the analysis. Also, a very thanks to my supervisor Prof. Peter Kille and all members of Professor Peter Kille's group in the School of Biosciences, Cardiff University, UK for their assistance and advice.

Special thanks to Prof. Taha A. Kumosani in Biochemistry department, Science faculty, King Abdul-Aziz University, Jeddah, Saudi Arabia for his support during the Jeddah sampling expedition and for the use of his laboratory facilities. This work was funded by Saudi Geological Survey via Ministry of Higher Education, Saudi Arabia.

Many thanks family and friends for all their encouragement. A special thanks to my parents for their unreserved support and encouragement throughout my life. Finally, my wife and my daughter for their unconditional support through all the ups and downs of this thesis.

ABSTRACT

The interface between urban combinations and associated industry with fragile ecosystems delivering significant ecosystem services denotes one of the critical frontiers for ecological genomic investigation. The major issue when evaluating diffuse pollution generated at this interface revolves around the possible interactions between mixtures of contaminates that individually remain below trigger level but together may result in significant environmental impact. To determine whether mixture effects need to be considered, it is essential to define geochemical parameters by performing a survey for major classes of contaminates and to evaluate their penetrance into the food chain. The coastal marine environment of the Saudi Red Sea is subject to direct and indirect influences of major populations and industrial facilities found along the coast such as those discovered in proximity to Jeddah City in Saudi Arabia. Sampling of both sediment and sea water was performed at contrasting sites representative of near-shore with off-shore locations. Possible food-chain transference of any contaminates was evaluated by sampling fish (*L. nebulosus*) and plankton at the off-shore sites. Biomarkers are mostly useful in the evaluation of progressive diseases that apparent their symptoms long after exposure to the initiating factor. In such cases, traditional early warning symptoms of developing disease may be lacking. Thus, detection of earlier events can provide a valuable timely warning of risk. It is important to identify and address the growing environmental problems being faced by the community and address it before it takes the shape of an epidemic. To assess toxicity of single and paired metals to the nematode *C. elegans*, toxicity tests were designed to first determine the impact of single metal exposure Copper, Zinc and Aluminium and then nematodes were exposed to paired combinations. Exposures with paired metals showed a variety of interactions which ranged from antagonistic to synergistic effects.

ABBREVIATIONS

µg	Micro gram
µL	Microliter
µM	Micromolar
AAPA	Amino acid permease
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAIBA	β-aminoisobutyric acid
CA	Concentration addition
CoA	Coenzyme A
Ctr1	Cu transporter 1
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FAD	Flavin adenine dinucleotide
FAME	Fatty acid methyl esters
g	Gram
GC-MS	Gas chromatography mass spectrometry
GSH	Glutathione
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSPs	Heat shock proteins
IA	Independent action
ICP-MS	Inductively coupled plasma mass spectrometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
kV	Kilovolts
LB	Luria broth

LC	Lethal concentration
min	Minute
ml	Milliliter
mM	Millimolar
MoA	Mode of Action
MTs	Metallothioneins
MUFA	Monounsaturated fatty acids
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NGM	Nematode Growth Medium
NMR	Nuclear magnetic resonance
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SPSS	Statistical Package for the Social Sciences
TCA	Tricarboxylic acid cycle (Krebs cycle)
TU	Toxic units

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 GENERAL INTRODUCTION

1.1 HUMAN ACTIVITY AND COASTAL ENVIRONMENT

In the past, one of the greatest attractions for the human habitation is the natural resources provided by oceans. But due to various human activities, these natural resources are at risk, threatening not only the destruction of key economic resources but also the disruption of the natural balance of the marine environment. In spite of the marine environment's natural grandeur being the source of imaginative inspiration, human activities are still a great threat to these resources and often result in the wasting of such resources (Icarus and Allen, 2011). Ultimately, these changes will impact on the water cycles and on the multitude of biogeochemical processes that are supported by marine ecosystems eventually leading to significant issues on a global scale (Duffus *et al.*, 2009).

Diverse mixtures of chemical pollutants together with biological waste and pathogens pose the greatest threats to both marine life as well as human health (PERSGA/GEF, 2003). Pathogens that are released into the oceans from waste water treatment plants may infect human hosts through the food chain or during bathing, surfing or other recreational activities. The potential pathogen-related health issues and related threats have been recognised by the US Environmental Protection Agency, resulting in stringent regulations (USEPA, 1997). The regulation of diffuse chemical pollution is a much more challenging issue (Shahidul Islam & Tanaka, 2004) and this is compounded by the absence of appropriate regulation.

It has been suggested that the deployment of widespread biomarker studies may provide an effective and fruitful evaluation of the damage inflicted by heavy metals on the marine environment (Shahidul Islam & Tanaka, 2004). In the past, various studies have extrapolated the results obtained from aquatic organisms' heavy metal body burden

as a bio-indicator of exposure impact and deduced the potential risks that can affect the health of the whole ecosystem. Appropriately selected biomarkers may better integrate the chemical and physiological changes caused by metal exposure on the organism representing additional valuable information when compared to the body burdens in the evaluation of the impact of heavy metal contamination (Montaser *et al.,* 2010).

For aquatic environments, a multitude of fish species have been employed as sentinel species providing indicators for the degradation of the aquatic environment. Sea anemones, sea urchins and grass shrimp were also used to determine the quantity of heavy metal toxicity in aquatic environments but ultimately the starfish species were chosen as the established sentinel species (Aspholm & Hylland, 1998). The quantification of fish metallothionein transcript levels serves two purposes; firstly, it acts as an early warning for degradation of environmental quality and secondly, it also allows for the specific measurement of the impact of specific subset of toxic and carcinogenic compounds (Evans *et al.,* 2000).

Proper pollution assessment techniques should be employed, not only for monitoring exposure to the present environmental contamination but also for notifying risk assessments. This requires the basic understanding of individual toxic chemicals and chemical groups and their associated biological responses. Bundy *et al.,* (2007) stated that endpoints that are easily measured, such as mortality, along with few sensitive tests including reproduction and growth are an integral part of eco-toxicological research. Also, it is of paramount importance to get a thorough understanding of the toxic mechanisms at a molecular level along with their relationship with the functional changes at the organism and population level (Spurgeon *et al.,* 2010).

Water uptake either via gills or skin (bio-concentration); uptake of the suspended particles in water (ingestion), and consumption of the contaminated food (bio-

magnification) are the major mechanisms that are responsible for causing the accumulation of persistent chemicals in aquatic organisms. Van Der Oost *et al.,* (2003) emphasizes the importance of bioaccumulation by highlighting the fact that it is among the hazardous principles despite bypassing or being unresponsive towards the standard eco-toxicity tests such as: relationships between biomarker responses and effects on pathology, survival, growth or reproduction. Yoon *et al.,* (2008) suggest that the Biota chemicals serve as an important determinant for adverse effects on ecosystems. Figure 1 clearly shows the processes which are involved in the bioaccumulation (uptake and clearance) model in aquatic organisms (Van Der Oost *et al.,* 2003). The uptake and elimination kinetics serve as the basic tools for the determination of contaminant levels in biota.

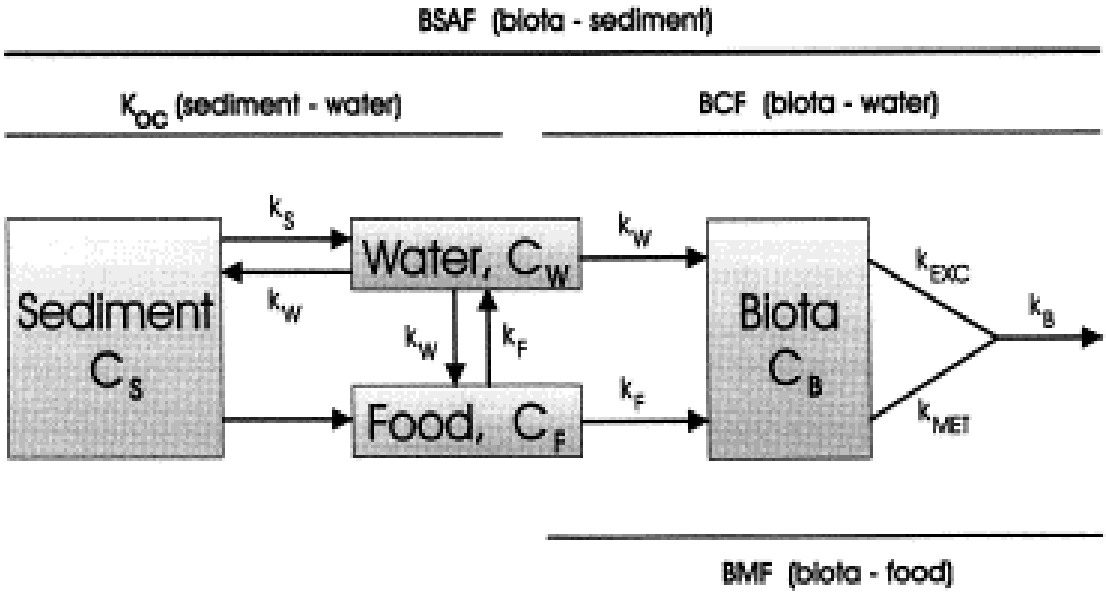

Figure 1: Bioaccumulation model for aquatic organisms. Where, KOC: sorption coefficient; BCF: bio-concentration factor; BSAF: biota-sediment accumulation factor; BMF: bio-magnification factor, C: concentration and k: rate constant. Among the subscripts: S= sediment, W= water, F= food, B= biota, EXC= excretion and MET= metabolism. The digestible sediment fraction is considered to be part of the food taken. Figure adapted from Van Der Oost *et al.,* (2003).

1.2 JEDDAH CITY AND RED SEA

Jeddah is the largest city in Mecca Figure 2, being second in size only to Riyadh in Saudi Arabia. The city is known as the main urban centre of western Saudi Arabia. With a population of over 3.5 million, Jeddah is the main gate to Islam's holiest city and a pilgrim once in a lifetime to this sacred place is mandatory for Muslims (Aleem, 1990). Municipal wastes (mixed sewage and industrial wastes) and port activities are one of the major causes of coastal discharge globally and are also a major threat to the Red Sea off the coast of Jeddah city, Saudi Arabia being responsible for both organic and metallic contaminants entering the coastal waters (El-ghazaly, 2006).

Figure 2: A map showing the location of Jeddah city on the coast of the Red Sea.

According to Trabulsi (2006), the quality of the local water is majorly affected by the intrinsic environmental impact of such a substantial urban centre with its associated heavy industry. Major desalination installations, oil refineries and a vibrant seaport are included in the list of the associated heavy industry. The basic aim of this research is to combine classical environmental chemistry with novel molecular ecotoxicology in order to address the risks that may threaten the sustainability of the local ecosystem in the Red Sea. Determination of any potential harmful impact on human health associated with contaminates in the Jeddah City is also a major objective of the study.

Various studies have been conducted on the coral disease in the Red Sea and have concluded that human activities are responsible for the majority of pollution especially oil and its derivatives (persistent carcinogens) accumulating in water and sediment (PERSGA/GEF, 2003). This study demonstrated that the coral reef health is majorly affected in the north of Jeddah compared to the south. Significant levels of diseases, especially Black Band disease, were reported to be the major cause of reef deterioration. Mohorjy & Khan (2006); and Massoud & Fahmy (1994) conducted experiments and concluded the desalination plant effluents, untreated domestic sewage wastes, by-products from oil refineries and biological wastes from the big fish market to be the major sources of pollution along the coast of Jeddah. Studies by Badr's group in 2008 and Montaser's group in 2010 also suggested that heavy metal pollution is the major threat to the local ecosystem of Jeddah's coast. The importance of Jeddah city for its geographic place, many of industrial and seaports activities and number of populations living there, makes it very interesting to investigate these activities impacts on the environment of this city.

1.3 HEAVY METALS

According to Dupont *et al.*, (2010), heavy metals, particularly transition metals such as Copper, Iron, Manganese or Zinc, are required as an essential cofactor by approximately 30% of all proteins encoded by the human genome. Metal ions are directly involved in metal-mediated control of gene expression Jackson *et al.,* (2008) and metal ion involvement in intracellular signalling either directly as explained by Yamasaki *et al.*, (2007) or indirectly as discussed by Evstatiev & Gasche (2012). Also, studies by Waldron *et al.*, (2009) state that the metallochaperones and metallotransporters proteins also possess specific independent functions useful for the body. For example, nickel is inserted into bacterial hydrogenase and urease by dedicated nickel metallochaperones.

Because most biological processes require metal ions as one of the basic elements for efficient functioning, tight control of homeostasis and all the associated activities of these metals are also required (Bertini & Cavallaro, 2008). If metal ion homeostasis is disturbed, the metals can either bind to undesirable sites or displace other correctly-bound metals from their respective sites (Nelson, 1999). The metal ion transporters are involved in maintaining homeostasis, and hence, the desired levels of metal ions in the body (Rolfs & Hediger, 1999). When these transporters fail to regulate the levels of metal ions, the homeostasis of metal ions is derailed, which results in the development of many of diseases due to the targeting of DNA and other proteins, and ultimately, causing oxidative deterioration of biological macromolecules (Halliwell and Gutteridge, 2007).

Some of the different mechanisms by which metals exert toxicity include blocking of important biological functional groups of biomolecules, the displacement of essential metal ions in biomolecules and the alteration of the active conformation of biomolecules. A well-known metal binding ligand, Metallothionein is not only synthesised during metal

exposure, is known to be synthesised in many different species, including invertebrates, in events of heavy metal exposure (Everett, 2001).

A study by Rand & Petrocelli, (1985) has found that Thionein synthesis is initiated after receiving relevant signals from metal ions such as Cadmium, Zinc, Copper, Mercury, Cobalt, Nickle, Bismuth and Silver. Also, the activity of the Zn-potentiated enzyme carboxypeptidase is known to be affected by the removal of Zinc but this activity can be restored to a considerable extent when Manganese, Iron, Nickle, and Cobalt are used in place of Zinc; all of which have a common binding site (Backhaus *et al.*, 2000).

Howell (1983), conducted a study on marine nematodes, *Enoplus brevis* and *Enoplus communis* by taking various tissues of the organisms and performing metal analysis to determine the pattern of heavy metal uptake and their respective distribution. He observed that these marine nematodes have pores or channels in the cuticle through which the rapid uptake and loss of Copper and Zinc occurs (Tatara, 1998).

Copper is an example of an important metal ion that is essential for multiple biological processes. The two oxidation states Cu (II) and Cu (I) are the most important form of Copper. It is not only an important factor in electron transport (Valko *et al.*, 2005) but also acts as a cofactor in different redox reactions such as cytochrome c oxidase, ascorbate oxidase, or superoxide dismutase. Direct formation of ROS via a Fenton-like reaction (Prousek, 2007) raised level of Copper which ultimately decreases glutathione levels (Speisky *et al.*, 2009). These two mechanisms led to Copper induced oxidative stress. As mentioned earlier, the Copper metal ion in the form of cupric (Cu^{+1}) and cuprous (Cu^{+2}) ions take part in oxidation reduction reactions. According to Aruoma *et al.*, (1991) the cupric ion Cu^{+2} has the capability to convert into cuprous ion Cu^{+1} by reduction, in the presence of superoxide anion radical or biological reductants such as ascorbic acid or Glutathione (GSH). This cuprous ion Cu^{+1} then catalyses the

decomposition of hydrogen peroxide via the Fenton reaction, and ultimately, results in formation of reactive hydroxyl radicals as shown in the following reactions:

- $Cu\ (II) + O_2{-}\bullet \rightarrow Cu\ (I) + O_2$ (1)
- $Cu\ (I) + H_2O_2 \rightarrow Cu\ (II) + \bullet OH + OH{-}$ (Fenton reaction) (2)

The reactive hydroxyl radicals then react further with the molecules that are in close proximity. Hydrogen abstraction leaves behind a carbon-centred radical or forms a lipid radical from unsaturated fatty acids. According to Moriwaki *et al.*, (2008), site-specific Fenton reactions are responsible for causing damage to the DNA and this DNA damage was more frequently caused by Copper (both Cupric and Cuprous forms) when compared with iron, particularly by the formation of the genotoxic benzene metabolite, 1, 2, 4-benzenetriol. Also, Copper is known to be involved in oxidation of bases via the production of ROS. Glutathione is a known powerful cellular antioxidant against Copper toxicity (Jomova & Valko, 2011) and a substrate for different enzymes which has been reported to remove the ROS. It is known to regulate Copper metabolism as well as its detoxification. According to Mattie and Freedman (2004), Glutathione chelates Copper, maintains it at a reduced state, and hence, prevents it from causing Copper toxicity. When GSH or Glutathione stores become depleted due to misbalancing of Copper homeostasis, a more oxidizing environment is created (Lee *et al.*, 2002). More ROS will be formed and in the absence of available GHS, Copper toxicity will occur.

Zinc is another important trace element and an antioxidant present in many plants and animals and constitutes one of the major components of the various enzymes involved in the metabolism of proteins, lipids and carbohydrates. It is a redox inert metal with +2 Zn (II) as its most stable oxidation state. Zinc acts as an antioxidant by ultimately reducing

the reactivity of sulphydryl groups. According to Nestor (2009), Zinc either provides protection to the sulphydryl groups of proteins against free radical attack or reduces the free radical formation particularly by opposing the redox-active transition metals, such as iron and Copper, to successfully perform its function as an antioxidant. Broadly, two processes are known to reduce or prevent the free radical or more precisely the hydroxyl radical formation. In the first process, the high-affinity ligand chelators are the major players that remove or "pull" off the metal ions from their binding sites. In the second process, a redox-inactive metal (e.g. Copper replacement by Zinc) performs a function similar to the chelator as it possesses a structure similar to the metal being replaced.

This redox-inactive metal then pushes and replaces the redox metal at its binding site (Stadtman, 1990). The detached metal then leaves the cell and, in this way, the undesirable oxidation reactions are bypassed. Jomova & Valko (2011) reports few classical examples of this Zinc antagonism mechanism which include the iron-mediated xanthine/xanthine oxidase-induced peroxidation of erythrocyte membranes, in copper–iron ascorbate-induced DNA strand breaks, the superoxide and hydroxyl radical from xanthine oxidase and NADPH oxidase and the Fe (III)-ascorbate-induced methemoglobin formation in red blood cells. Various models were also put forward to explain how Zinc shows its antioxidant activity. ROS may damage critical processes in the cell and lead to a plethora of toxic consequences. However, cells have an extensive endogenous defence system against this hazard. It consists of antioxidant enzymes, such as Superoxide dismutase (SOD), Chloramphenicol acetyltransferase (CAT), and Glutathione peroxidase (GPX) and of small antioxidants, such as Glutathione (GSH), Metallothionein (MT), Vitamins C and E. Glutathione S-transferase (GST) and Glucose-6-phosphate dehydrogenase (G6PD) enzymes help in the detoxification of reactive oxygen species by decreasing peroxide levels (GST), or by maintaining a supply of reducing power in form of Nicotinamide adenine dinucleotide phosphate (NADPH) (G6PD). Moreover, there are

indications that exogenous antioxidants like ascorbate and α-tocopherol offer some degree of protection against this type of DNA damage (Chung *et al.*, 2005).

In these models, it was briefly suggested that either Zinc binds directly on the sulphydryl group, or in close proximity to the sulphydryl groups in a different binding site or in a site other than binding site which results in conformational changes in proteins, and hence, reduces reactivity of sulphydryl groups. Many sulphydryl containing proteins like dihydroorotase, DNA Zn-binding proteins (Zinc fingers) and protein farnesyltransferase are reported to be protected by the antioxidant activity of Zinc (Jackson *et al.*, 2008). Heavy metals that produced from industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment. Their toxicity depends on several factors including the dose, route of exposure, and chemical species. For that we are focusing in this study to investigate heavy metals impact on the health using a chosen organism as a model.

1.4 METAL MIXTURE REVIEW

The metals that contaminate the environment are usually present in the form of metal mixtures, containing toxic metal cations. According to (Pagenkopf, 1983), at the level of biotic ligands H^+, Na^+, K^+, Ca^{2+} and Mg^{2+} compete with toxic ions, such as Cu^{2+} and Zn^{2+} for binding sites. This competition may results in reduced uptake of toxic metals, and hence, in metal toxicity. Cations are known to be directly associated with metal toxicity. Little research has been done into the contribution of the ion-ion interactions in predicting bioaccumulation and toxicity of mixtures of toxic metals (Borgmann, 2008). When large amount of cations are present, they decrease the negative charge of the electric potential at the plasma membrane (PM) surface, which not only affects the

activity of toxic ions at the PM surface but also hinders effective transportation of various ions through the membrane (Kinraide, 1999).

According to Kabata-Pendias (2000), the interaction between the metals in the mixtures may occur at the environment level and when these interactions taking place at the environment level are considered, it can be seen that the magnitude of the interaction depends upon the amount of metal ions present in the environment. The physicochemical conditions of the environment also play an important role in initiating possible interactions. But when the interaction occurs between the metal components in lower quantities, they can result in serious adverse effects (Cooper *et al.,* 2009). The harmful effects of these interactions show themselves both toxico-kinetically and toxico-dynamically by affecting uptake due to interaction among different substances and by affecting the combined toxicity due to target site interaction in organisms, respectively. Kalis & Temminghoff (2006) states that the competition between different ions greatly affects the metal binding and their uptake. These metals ions also compete for the binding sites during the absorption, excretion, or sequestration phases even after accumulation (U.S. EPA (Environmental Protection Agency) EPA/635/R-05/002, 2007).

A study showed that at the organism-water interface, such as fish gills, the competition for binding sites usually occur between the H^+, Na^+, Ca^{2+}, and Mg^{2+} cations and the toxic metal ions such as Cu^{2+} (Pagenkopf, 1983; Zitko & Carson, 1976; Campbell *et al.,* 2002). Similarly, Cu^{1+} and Ag^{1+} can compete for the transporter Ctr1 (Boyle *et al.,* 2011). Borgmann (2008) in his study points towards a major threat related to the hazards associated with the metal mixture interactions. He suggests that more hazardous combined toxicities of an additive, synergistic, or antagonistic nature can result when there is interaction among different metal ions as well as the interaction between metal

ions and cellular systems that includes uptake, transport and receptor-binding, which occur at the same time.

One of the biggest challenges faced by the environmental scientists, risk assessors and regulators is the determination of potential toxic effects as well as the proper quantification of the risks associated with these toxic activities on the environment and the human species. Understanding a framework along with standardised testing procedures is required to achieve this purpose. One of the most widely accepted frameworks, the paradigm of additively (Additive interaction means the effect of two chemicals is equal to the sum of the effect of the two chemicals taken separately. This is usually due to the two chemicals acting on the body via same or similar mechanism) is being utilized to determine the combined effects of different chemicals (Spurgeon *et al.*, 2010). Depending upon the mode of action, two models constitute this framework. The first is the concentration addition (CA) model for chemicals with the same Mode of Action and the other one is the independent action (IA) model for chemicals with different Mode of Action (Cassee & Groten, 1998). The ecotoxicological testing of both simple and complex mixtures of compounds which have a similar Mode of Action (Altenburger, 2010) and dissimilar Mode of Action, can be performed using these models.

Neither the CA or the IA models mentioned are applicable in all cases (Jonker *et al.*, 2005; Bundy *et al.*, 2008) but these models serve as a mathematical basis for the prediction of mixture effect as either quantitative descriptors of the relative toxicities of the individual chemicals in the mixture (CA) or the relationships between the probabilities of response under an assumption of wholly independent effects (IA) shown in Figure 3.

To determine the interactions, three types of mixture models have been provided (Ribeiro *et al.*, 2011), which are based on the analysis of response curves. This study suggested that when the toxicants perform independently and have different modes-of-

action, independent combined action occurs. According to Bliss (1939), every single component has the capability to synergize or antagonize the action of the other component. Therefore, when the effectiveness of the mixture depends upon the combined toxicity of different amounts of toxicants, synergistic (that is the creation of a whole that is greater than the simple sum of its individual components) action occurs.

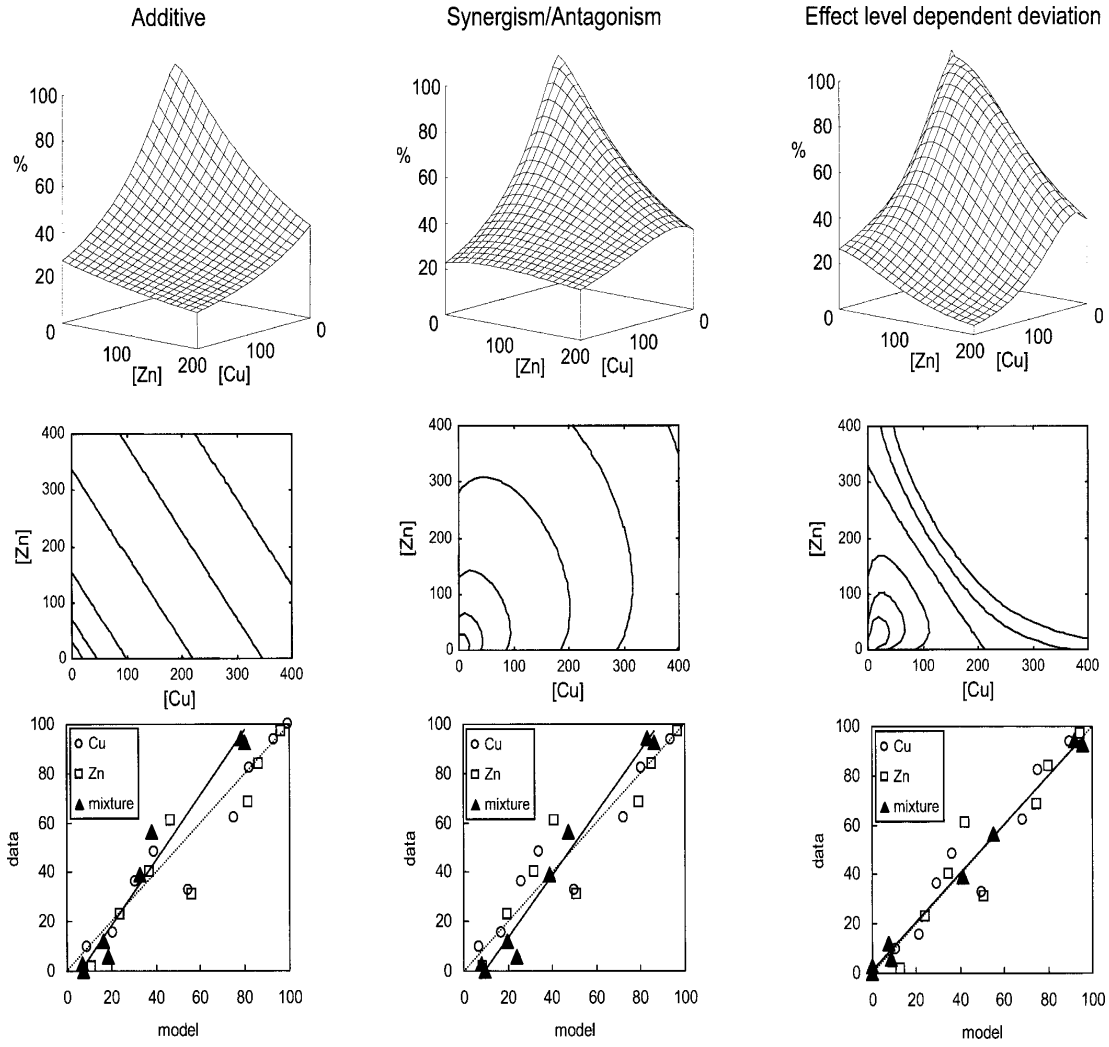

Figure 3: The additive model showing two deviation patterns: synergism/antagonism and effect level–dependent deviation. The effect of Copper and Zinc (µg/g dry soil) on the proportional number of extracted nematodes is shown by the Response planes (top row). Isobolic representation of the models is shown in the middle row. The isoboles indicate 10, 25, 50, 75, 85, and 90% effect from left to right. (Concentrations in µg/g dry soil). The bottom row shows comparisons of the model description with the data. The dotted diagonal lines indicate ideal model description whereas a regression analysis over the mixture data is represented by the solid lines. Each point represents an average of four replicates to the minimum (Jonker *et al.*, 2004).

Biomonitoring tools such as emission patterns for certain geogenic conditions or for specific processes such as mining or smelting and by-products such as biocides on ship hulls, are known to be capable of detecting the concentration of metal ions in the environment, despite the fact that metal ions in the form of charged species are not easily taken up by organisms. According to Altenburger (2010), different biomonitors can prove very useful in the determination of body burden or internal exposure of organisms to multiple metal compounds which are being released in the environment as a result of various anthropogenic activities.

In Figure 4, a framework is shown, illustrating both the theories of external exposure or 'bioavailability' of the mixture in the environment along with its exposure in the respective species (Spurgeon *et al.,* 2010). Both the accumulation of metal toxins through toxic kinetics and toxic dynamics are also demonstrated in Figure 4.

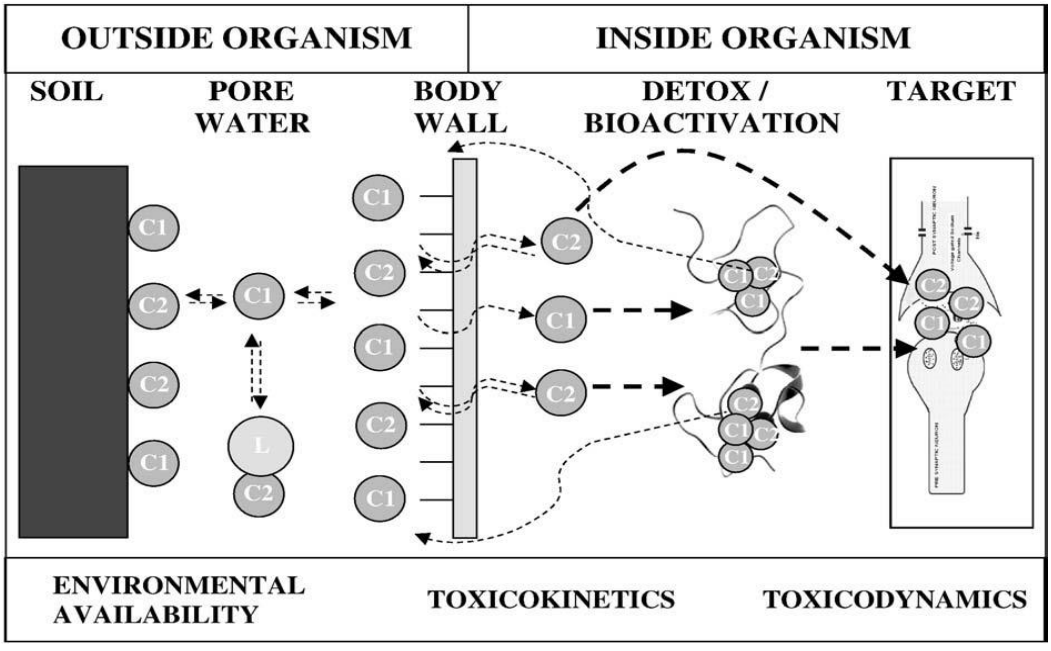

Figure 4: A framework illustrating metal mixtures effect along with the key role of toxicokinetics and toxicodynamics. Explains interactive effects in mixtures highlighting the importance of process involved in determining external exposure, toxicokinetics and toxicodynamics. Figure adapted from Spurgeon *et al.,* (2010).

These metal mixture interactions are not generalized and they happen in the same way in all species. In fact, these interactions occur differently in different organisms. They may be antagonistic in one species and additive in the other or *vice versa*. Therefore, for better understanding of these metal mixture interactions along with the corresponding harmful effect they produce, it is necessary to carry out more specific evaluations. Sensibly designed experiments to test the hypotheses relative to metal uptake can serve as a helpful tool in this regard for example, Metabolomics and Ionomics.

1.5 METABOLOMICS

To get a thorough understanding of the metal toxicity is quite a challenging task. A number of studies have been carried out on specific microbes such as yeast *Candida* and higher organisms (Booth *et al.,* 2011). There is still a long way to go before a complete understanding of the complex mechanism of metal mixture toxicity along with its corresponding adverse effects is gained. Different tools have been provided by the modern technology in this regard. A recent modern technology, 'Omics' provides a system-wide view of the changes that occur in cell physiology under stressful conditions. Metabolomics is one such technology that has promised a better and deeper study of metal toxicity along with the provision of a unique perspective on metal-induced changes in cellular metabolic architecture (Booth *et al.,* 2011).

The technology of metabolomics makes the study of physiology at the whole organism level much easier and provides quantitative information about the highest levels of functional components within cellular processes. The major aim of this technology is the systematic identification and quantification of the compounds called metabolomes. Metabolomes are defined as the cell's complement of small, low molecular weight compounds, the metabolites which are essential for growth, function and maintenance of

the cell. According to Booth *et al.,* (2011) amino acids, nucleotides, carbohydrates and lipids constitute the major classification of metabolites.

Metabolomics is a new discipline and is a sub-discipline of environmental toxicometabolomics. Being a novel discipline, there is so much yet to be explored. A few relevant studies that have been done in the area of metal toxicity include the analysis of the effects of cadmium in different plants; two *Pseudomonas* species in the presence of metal stress; the effect of metal contaminated sites on earthworm; the effects of various metals in rodents and; the model organisms *Caenorhabditis elegans*, *Daphnia magna* and *Gasterosteus aculeatus* (Kakkar & Jaffery, 2005; Booth *et al.*, 2011), respectively.

The fundamental parameters that are known to be of paramount importance in toxic metal interactions are the metal concentration, the exposure conditions as well as the presence of certain media components. The important exposure conditions that can alter cellular metabolism include time and adaptation. This applies to both macro- and micro-organisms but the detection of metabolites is much easier in case of macro-organisms as large sample numbers are needed in cases involving microorganisms. According to Booth *et al.,* (2011) it is a very challenging task to acquire enough cell mass, especially from a slow-growing bacterium, to use on a high sensitivity platform such as Gas Chromatography Mass Spectrum (GC-MS).

According to several metal toxicity studies, some toxic metals, such as aluminium are reported to evoke significant metabolic shifts in the cell (Vaidyanathan 2005). Metal stress is known to be one of the major factors involved in causing significant metabolic changes in the cells of various organisms. Metabolic pathways and other cell physiology were observed to be altered in the metal-exposed microbes during different transcriptomic and proteomic studies (Lemire *et al.*, 2008). In one study, mycelia formation and biofilm differentiation were reported to be affected when yeast *Folsomia candida* was exposed to

different metals (Baas *et al.,* 2007). Systems biology approaches in toxicology offer an extraordinary potential to unravel the complex effect pathways of chemical contaminants and significantly enhance our understanding of their effects on the health of organisms. In concert with phenotypic measures of chemical exposure and health effects, the application of "omics" techniques has provided the opportunity to distinguish alterations associated with adverse health effects from those involved in normal acclimation to changing conditions. Such information is also value in the identification of appropriate biomarkers of exposure, able to act as early warning systems for the protection of environmental and human health.

1.6 AMINO ACIDS

Amino acids, osmolytes and intermediates of the Krebs Cycle Figure 5 are known to suffer significant metabolic changes when organisms are exposed to Copper. Copper is reported to cause disturbances in osmotic regulation and energy metabolism in Manila clams, *Ruditapes philippinarum*. When clam gills were exposed for 96 hours in Zhang's 2011 study, it was observed that Copper results in increases in homarine and branched chain amino acids, whereas succinate, alanine and dimethylamine levels decreased. Toxicological mechanisms of marine heavy metal contaminants can be monitored by using metabolic biomarkers by the application of the Nuclear magnetic resonance (NMR)-based metabolomics (Zhang *et al.,* 2011).

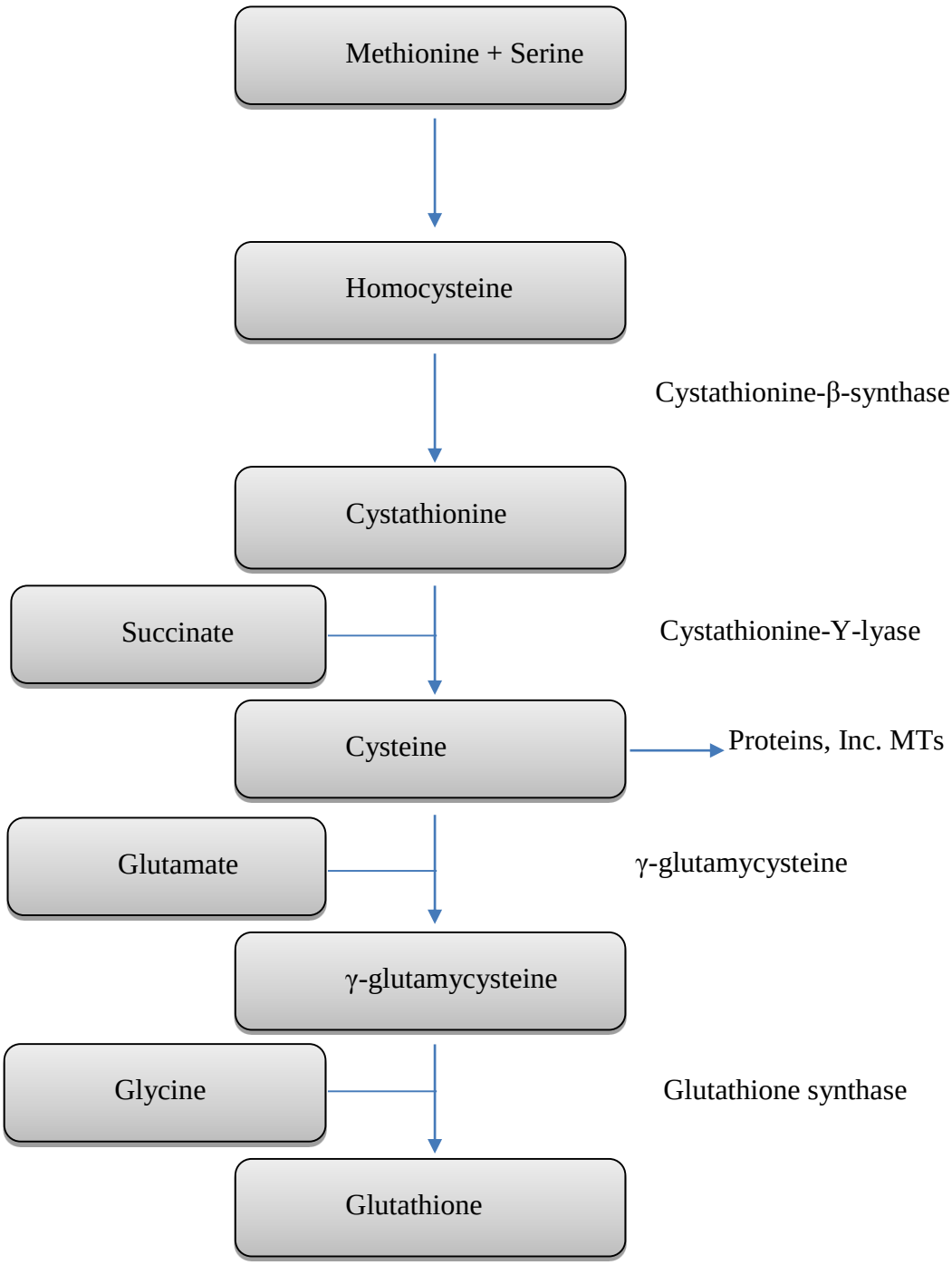

Figure 5: Metabolic interconversions in *C. elegans*. Metabolic interconversions in *C. elegans*. Cystathionine, a key intermediate in the transsulfuration pathway, was significantly decreased in concentration following cadmium exposure. This is due to the inhibition by cadmium at the free thiol groups of two enzymes Cystathionine-*β*-synthase (C*β*S) and Cystathionine-γ-lyase (CγL). There is consequently an increased flux of cysteine into glutathione synthesis and phytochelatin (Hughes *et al.*, 2009).

When different samples of Manila clam, *Ruditapes philippinarum* were exposed to both high and low doses 10 and 40 μg L^{-1} of Copper for 48 hours, specific metabolic responses were observed which included an increase in branched chain amino acids and glycine levels and a decrease in the ATP/ADP ratio. Therefore, the increase of amino acids in clam gill tissues was actually an indication of Copper toxicity (Zhang *et al.*, 2011). According to Viant *et al.,* (2003), high intracellular concentrations of free amino acids are being utilized by some marine molluscs for cellular energy metabolism and also to balance their intracellular osmolarity with the environment.

1.7 FATTY ACIDS

In order to explore the genetic basis of fatty acid synthesis along with the regulation of fat storage, the nematode *Caenorhabditis elegans* is the best choice (Mullaney & Ashrafi, 2009). Not only the genetic basis of fatty acid synthesis, regulation of fat storage and energy homeostasis but also the relationship of metabolic processes of the organism with growth, reproduction and lifespan can be thoroughly investigated though special powerful genetic and behavioural tools (Kakkar & Jaffery, 2005).

The most fundamental hydrophobic components of all the cell membranes are the fatty acids. Selective permeability, membrane fluidity and signalling are one of the major functions of these fatty acids. The *C. elegans* either manufacture their own fatty acids by *de novo* synthesis using acetyl CoA or obtain it from their bacterial diet. *C. elegans* possess two multi-functional enzymes, the acetyl CoA carboxylase and fatty acid synthase Rappleye *et al.,* (2003), which are destined to synthesize palmitic acid (16:0). Then after passing through a number of steps, acetyl CoA successfully forms different fatty acids, which includes many long-chain polyunsaturated fatty acids (PUFAs) and

monomethyl branched-chain fatty acids (mmBCFAs) (Kniazeva *et al.*, 2004). The long-chain polyunsaturated fatty acids (PUFAs) are formed by palmitic acid in the presence of fatty acid elongases and desaturases Figure 6a whereas the monomethyl branched-chain fatty acids (mmBCFAs) are produced from branched-chain CoA primer Figure 6b. These fatty acids are considered to be essential for proper growth of the organism.

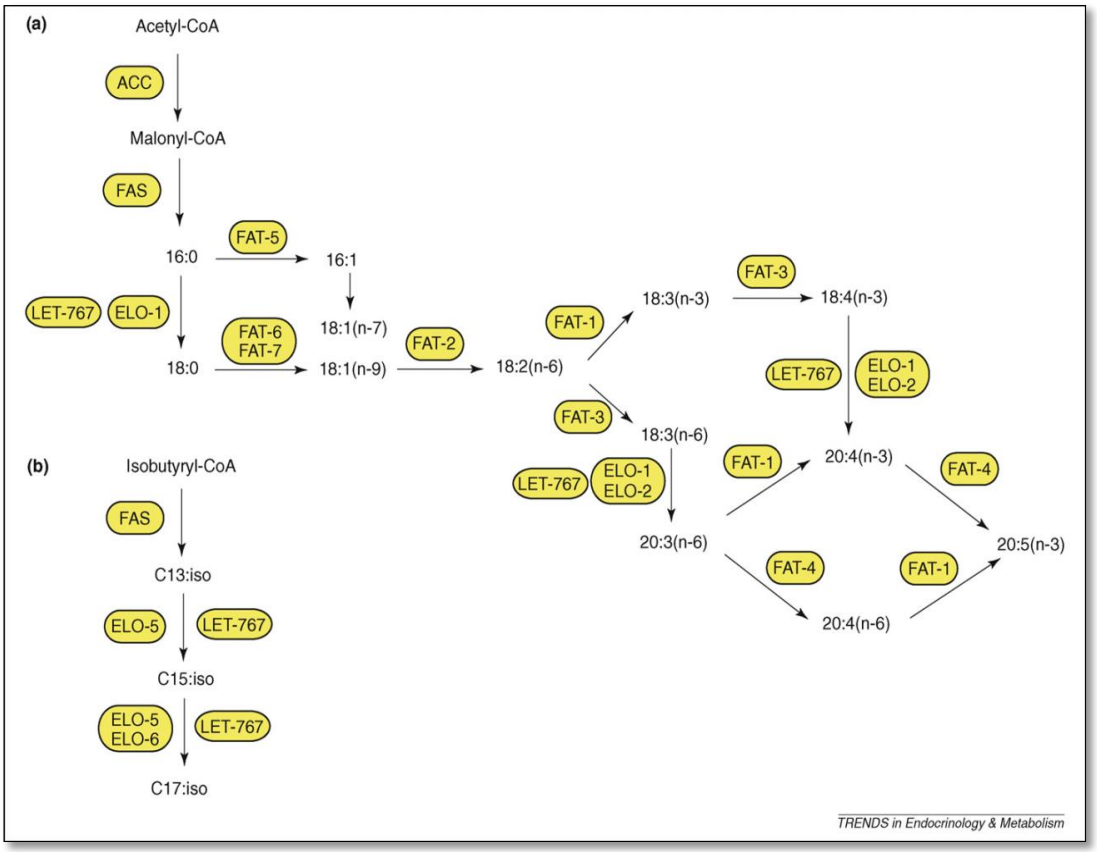

Figure 6: Fatty acid synthesis pathways in *C. elegans*. (a) *De novo* synthesis of polyunsaturated fatty acids (PUFAs) is shown, which starts with acetyl-CoA and utilizes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Dietary fatty acids such as palmitic acid (16:0) may enter the pathway and in the presence of various desaturase and elongase, they get converted to PUFAs. (b) Monomethyl branched-chain fatty acid synthesis is shown which uses a branched-chain primer as a substrate. The FAS and specialized elongase form two essential growth factors; the C15: iso and C17: iso. In the Fatty acid nomenclature, 'X: Yn-Z' indicates the fatty acid chain of X carbon atoms and Y methylene-interrupted *cis* double bonds and Z indicates the position of the terminal double bond relative to the methyl end of the molecule. Gene activities are: FAT-1, omega-3 desaturase; FAT-2, D12 desaturase; FAT-3, D6 desaturase; FAT-4, D5 desaturase; FAT-5, D9 desaturase; FAT-6, D9 desaturase; FAT-7, D9 desaturase; ELO, fatty acid elongase; LET-767, 3-ketoacyl-CoA reductase. Figure taken from (Watts, 2009).

In order to determine the contribution of dietary fat absorption and *de novo* synthesis to fat stores and membrane lipids in *C. elegans*, a technique using ^{13}C isotope was developed by (Perez & Van Gilst, 2008). This technique was found to be very helpful in the quantification of fat synthesis. In the ^{13}C isotope assay, a mixture of ^{13}C-enriched *E. coli* and unlabelled *E. coli* were fed to the nematodes. Using gas chromatography mass spectrometry, the nematodes were then analysed and their triglycerides and phospholipids levels were determined. It was observed that the relative proportion of absorbed versus synthesized molecules varies depending on the type of fatty acid. Most of the palmitic acid (16:0) was directly taken up from the bacterial diet whereas much less, only 7%, was synthesized by the organism itself. On the other hand, when the same wild type strain N_2 *Caenorhabditis elegans* (Girard *et al.*, 2007) nematodes were observed for the C18 monounsaturated fatty acids (MUFAs) and PUFAs, it was found that most of the fatty acid was formed by *de novo* synthesis. Similarly, *de novo* synthesis was also the major process in case of mmBCFAs C15 iso and C17 iso with the percentage as high as 99%. This was primarily due to the absence of these fatty acids (mmBCFAs C15 iso and C17 iso) in the bacterial diet. Both the phospholipid and TAG fractions were found to have different dietary fatty acids including 16:0, 16:1 and 18:1n7. According to Watts (2009), similar amounts of synthesis and absorption of these fatty acids are demonstrated by the ^{13}C isotope assays.

1.8 IONOMICS

The study of elemental accumulation in living systems that make use of the high-throughput elemental profiling is termed as Ionomics (Baxter, 2010). Forward and reverse genetics, screening diversity panels, and modelling of physiological states in plants are some of its applications.

In order to understand the proper working of the living system, it is mandatory to understand the functions and dynamics of different elements, nucleic acids, proteins, and metabolites that act as essential building blocks of the living body. Ionomics analysis is found to be an attractive profiling technology by many researchers due to some of its most distinguishing properties. Firstly, the Ionomics analysis is a highly cost-effective technique. Sample throughput of this technique is also very high which is ideal for genetics and modelling studies. Several hundred samples can be run per day on an ICP by the help of an auto-sampler. Also, Ionomics analysis provides a comprehensive approach and allows simultaneous measurement of most of the relevant ions.

Trace elements are known to be required by the body for efficient functioning. An element in a sample that has an average concentration of less than 100 parts per million (ppm) atoms, or less than 100 micrograms per gram is known as a trace element in analytical chemistry. Geochemistry says that a trace element is a chemical whose concentration is less than 1000 ppm or 0.1% of a parent rock's composition. According to Kabata-Pendias (2000), a trace element or micronutrient is a chemical element that is needed in very small quantities for the efficient growth, development, and physiology of the organism. Different processes like reduction and oxidation reactions are known to allow the entry of these trace elements into plant and animal systems. The trace elements are required up to a certain limit by the body for regular functioning. When the quantity exceeds the normal limit, toxicity occurs. Not only the quantity of trace elements is responsible for their toxicity but the trace elements may also acquire some toxic properties by alteration of elemental stoichiometry by various human activities. According to Leggat (2011), the definition of a 'xenobiotic' is a substance not produced by the body but are present in the environment before entering the biological system.

Different trace elements, from vanadium to zinc in the first row series of the periodic table, plus molybdenum in the second row series, are essential for the efficient functioning of both plants and animals. According to (USEPA, 1988), the physiological range for these elements becomes narrower between sufficiency, deficiency and toxicity and requires the presence of a tightly controlled metal homeostasis network for the adjustment of fluctuations. A study in southeast Missouri to monitor the effects of trace elements on the ecosphere observed the perturbations to the litter–arthropod food chain in a forest ecosystem as a result of the exhausts of heavy metals from a lead ore-processing complex (Dudka & Adriano, 1997). The litter is generally used as food by the arthropods and is an important part of the food chain in a forest ecosystem. When such litter containing high concentrations of Lead (Pb), Zinc (Zn), Copper (Cu), and Cadmium (Cd) was consumed by the arthropods, it resulted in a decrease in arthropod density and microbial activity. This ultimately caused a decrease in the rate of decomposition, and hence, disturbance of forest nutrient dynamics.

According to Shanker (2008), when the researcher have enough information about the Mode of Action of metal toxicity, that knowledge can then very helpful in the following tasks:

(a) For the interpretation of toxicity data,

(b) For estimation of both the probability as well as the extent of harmful effects,

(c) For protocol development to prevent toxic effects, and

(d) For discovering the antidotes or drugs that can counteract these toxic effects.

One of the benefits of working with inorganic elements is that the common size and chemical properties give them the advantage that they are relatively inexpensive to measure, since a large range can be measured in a single analytical run (Baxter, 2010).

The greater the number of elements measured in a single experiment, the greater the test's accuracy and power no matter which statistical treatment is being used. Baxter *et al.*, (2008) showed that for determining the physiological state of the growth medium, measuring multiple element Fe and P replicates were an improvement when compared to measuring the individual elements. The reason being is the study cannot be biased by atmospheric or random changes in a single element. Some modified plants can distinguish among some analogues, however. The analogues include, S/Se, Ca/Sr and K/Rb are considered in one class of element connections Figure 7 (Baxter, 2010).

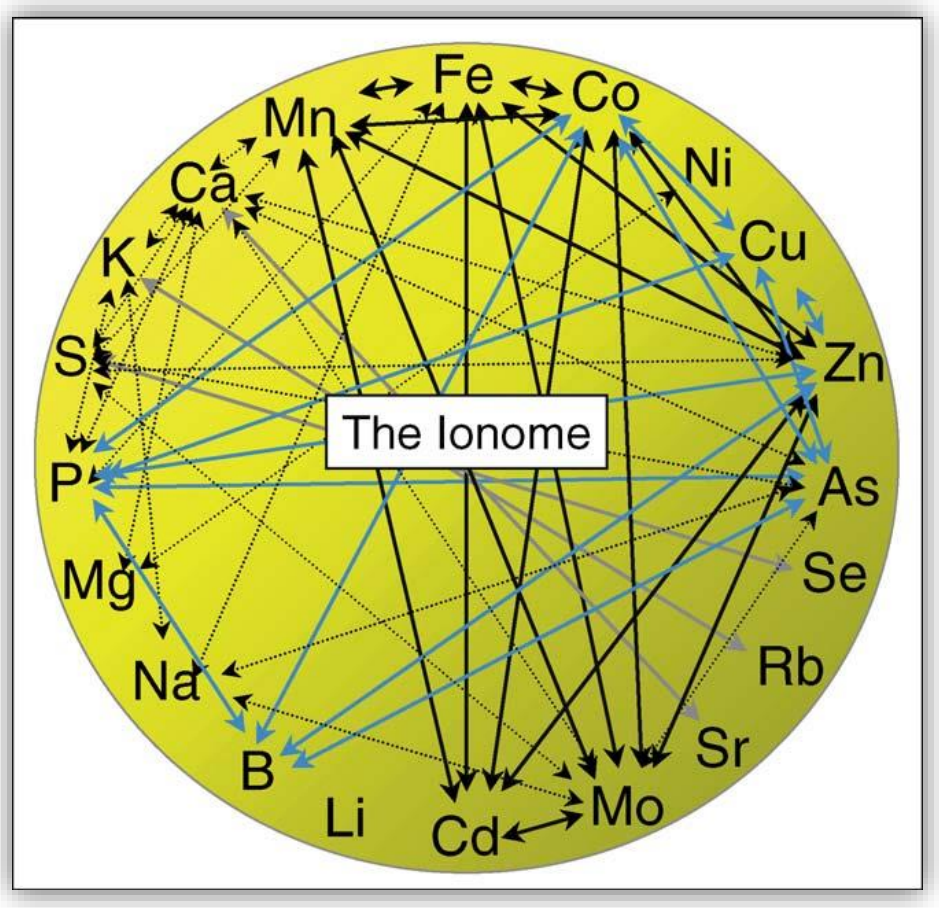

Figure 7: Metal interactions described in Baxter's article are indicated through arrows. The diagram details the acknowledged elemental interactions. There are physical, chemical and genetic interactions taking place between the elements. Chemical analogues are shown through grey arrows (Baxter, 2009), while black arrows indicate Fe model elements, and lastly, the blue arrow is the P model elements from (Baxter *et al.*, 2008).

A number of studies have been carried out to observe the effects of different elements on the human body including one on the effects of exposure of Aluminium. Aluminium has higher binding affinity in comparison to physical binding partners and it is for this reason Aluminium interrupts the balance of Iron and Calcium along with other metals. However, through research, it has been determined that there is some connection between Aluminium exposure to some types of dementia, osteomalacia and microcytic anaemia (Becaria *et al.,* 2002). However, the molecular mechanisms underlying such disorders are undefined. Possibly, the altered metabolism (the metabolites that are altered by cancer cells) may produce higher attraction of Aluminium over Magnesium for ATP and free phosphate, allowing the possibility for Aluminium to intervene with Mg-catalysed enzyme activities (Page *et al.,* 2012). Transformed elemental homeostasis at all levels of complexity are associated with a higher level of Aluminium according to previous research (Yang *et al.,* 1998). In a few biological systems, Aluminium alters homeostasis of Copper (Williams, 1999). During the study of the linkage between toxic pathology and individual metals, the effect of toxic challenge on the metallome (distribution of free metal ions in every one of the cellular compartments) should be considered. We hypothesise that global metal homeostasis is altered by Aluminium because Aluminium exposure affects the level of different metals in a range of species and several pathologies also arise due to Aluminium exposure. It has been assumed that owing to a difference in the levels of some key elements, like Phosphorus (P), Copper (Cu), Sulphur (S), Sodium (Na), and Iron (Fe), an elevated Aluminium (Al) can result in disturbance of metallostasis (Page *et al.,* 2012).

According to Shanker (2008), a study was conducted by Voegtlin *et al.,* (1923) in which he studied the mechanism of action of Arsenic on the protoplasm. Previously, the major focus was diagnosis and treatment of the metal related toxicities or investigation at the clinical level. The focus then shifted towards long-term investigations at the level of

cells and molecules with the advent of modern sciences. A model which gained much importance in the 1970s due to its capability to allow the prediction of metal toxicity was based on pH and water hardness (USEPA, 1997). Algae and other aquatic organisms were analysed on the same principle as the model in which the influence of pH and water hardness on metal toxicity was determined. Nowadays, plants, micro-organism and higher animals are under detailed study to investigate their interaction with the trace elements and their corresponding harmful effects.

1.9 *CAENORHABDITIS ELEGANS* NEMATODE

C. elegans are 1 mm long nematodes and are free-living Figure 8. As they are mostly self-replicating hermaphrodites, they do not experience genetic drift making them a useful laboratory model and they have great importance in the field of biology and toxicology. *C. elegans* is kept in stocks at The *Caenorhabditis* Genetics Centre at the University of Minnesota for the purpose of supplying the scientific community.

C. elegans nematodes can be easily maintained on agar along with an *Escherichia coli* bacteria lawn (Brenner, 1974). As they are small in size, the aquatic experiments can be performed with small volumes (1 ml for 10 nematodes) allowing for little waste. Using liquid media or agar plates, thousands of nematodes could be grown (Kenyon *et al.*, 1988). After the juveniles hatch out of the eggs, it goes through four phases of life to attain its maturity Figure 8. Around 3.5-4 days in 20^{o}C, the fourth moult occurs after which the adult is now fertile and will live for a further 15 to 20 days. A dissecting microscope is used to study and observe the organism.

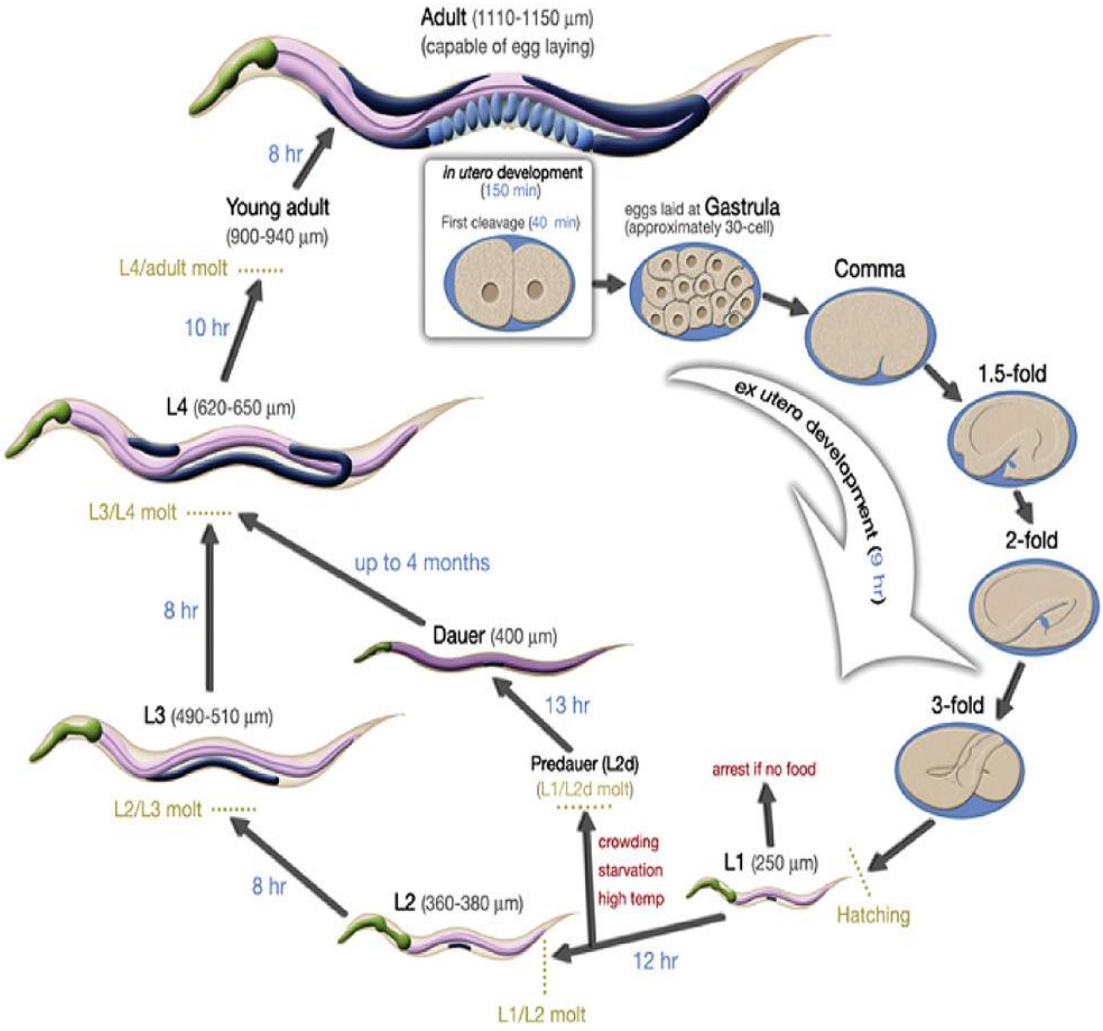

Figure 8: *C. elegans* life phases in 20 ^{0}C. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40 min. Eggs are laid at about 150 min. The length of the animal at each stage is marked next to the stage name in micrometres. Figure adapted from Baldwin *et al.*, 2011.

The use of *C. elegans* has increased in recent years, especially in neurobiology, genetics and developmental biology. It was the first multicellular organism to have its entire genome sequenced in 1998 (Equence & Iology, 1998). It has also been involved in the genomes list which is vital for human genome project as they help in decoding human sequence. The detailed understanding of its nervous system and genome along with its structural simplicity could be the main reason for its use (Equence & Iology, 1998).

There is a similarity in the pathways used by humans and nematodes to develop small molecules, store energy and decomposition of nutrients. Supporting evidence for this was obtained from the resemblance a nematode genome has to the human with respect to the presence of functional motifs in proteins (Braeckman *et al.*, 2009). Certain specific proteins are responsible for the basics of cellular respiration that include pyruvate decarboxylation (formation of acetyl-CoA from pyruvate), the citric acid cycle, ATP synthesis, glycolysis (the conversion of glucose to pyruvate), β-oxidation of fatty acids and the electron transport chain. The related protein for most potentially conserved genes is found to be operative and expressed. *C. elegans*, similar to mammals, uses Flavin adenine dinucleotide (FAD) and Nicotinamide adenine dinucleotide (NAD) for the purpose of cellular respiration and the proteins involved in this process are highly conserved. Adult nematodes have about 3.3% of glycogen dry mass in their bodies. The nematodes also store glucose and lipids in their bodies as an energy source and they have the ability to convert glucose into glycogen (Hulme & Whitesides, 2011). Adult nematodes are composed of approximately 36% dry mass of free fatty acids and triacylglycerides .The gut is the main location for the storage of glycogen. *C. elegans* does not have glucose-6-phosphatase in their bodies which help in the breakdown of glycogen into glucose. Therefore, they make use of trehalose for the process of conversion of glucose into glycogen. The body of nematodes contains certain enzymes that accelerate the process of conversion of trehalose from glucose-6-phosphate and also enzymes that enhance the hydrolysis of trehalose to glucose (Hulme & Whitesides, 2011).

The species of *C. elegans* is a well-established experimental model and a large body of data has confirmed that it has the majority of enzymes utilised by the main pathways of intermediary metabolism, as detailed in databases such as Nematode Base®, Reactome® and KEGG®. However, this does not confirm the concerted action of these enzymes in these biochemical pathways. Gene expression analysis, enzyme studies and

techniques for quantifying metabolites are required for understanding the activities of these biochemical pathways.

In 1963, Sydney Brenner proposed research into *C. elegans* primarily in the area of neuronal development and then in 1974 he began research into the molecular and developmental biology of *C. elegans*, which has since been extensively used as model organism. *C. elegans* uses similar energy pathways as humans involving sugars, amino acids and lipids to metabolise energy under aerobic conditions. In case of sugar or particularly glucose, the process of glycolysis is used to produce energy. The sugar is first converted into pyruvate along with ATP in the presence of different enzymes. At the same time, the electron carriers also get loaded with electrons (NAD^+ is reduced to $NADH^+$). The pyruvate then goes to mitochondria where it is converted to acetyl-CoA and CO_2; to be released by the process of decarboxylation. Different oxidation reactions occur which ultimately completes the Tricarboxylic acid cycle (TCA) cycle by forming oxaloacetate, along with expulsion of two carbons as CO_2, formation of (G/A) and reduction of electron carriers (NAD^+ and FAD^+) during the cycle. This is followed by sequential electron transport chain redox reactions taking place in the redox proteins embedded in the inner membrane of mitochondria. In these redox reactions, the electron carriers that were reduced during glycolysis and the TCA cycle deliver their electrons to O_2 accompanied by the formation of water. This transference of electrons results in the development of an electrochemical gradient; the potential energy of which is utilized by ATP-synthase, a protein complex in the inner membrane of mitochondria. This ATP-synthase then initiates the process of ATP synthesis (Braeckman *et al.,* 2009).

C. elegans possesses a functional methylmalonyl-CoA epimerase (racemase) (Kühnl *et al.,* 2005), which is known to be involved in propionyl-CoA metabolism for the

degradation of branched amino acids and odd-chain fatty acids. Different pathways take part in breaking of amino acids, the residual carbon skeleton of which is used in the TCA cycle. In the case of lipids, the process of β-oxidation causes the breakdown of fatty acid moieties of lipids into acetyl-CoA along with the formation of reduced electron carriers; β-oxidation takes place in the mitochondrial matrix. The acetyl-CoA formed as the end product of β-oxidation also enters the TCA cycle. It is important to note that among the usual energy metabolism pathways, the peroxisomal β-oxidation of long-chain fatty acids is not included though it is indirectly linked to energy metabolism.

On the other hand, mammals have enzymes in the liver that have the ability to decompose glycogen into glucose. This glucose then flows in the blood to supply the body tissues with the required energy. It has still not been understood which of the compounds, glucose, trehalose or both transfers the energy to the tissues by diffusion and decomposition of the glycogen in *C. elegans*. There exist a few more similarities in between *C. elegans* and mammals such as *C. elegans* rely on anaerobic glycolysis when they require energy under anaerobic conditions, which is exactly the case for humans. *C. elegans* produce a waste product called lactic acid when relying on anaerobic glycolysis. Among the differences that have been observed, one difference is that the *C. elegans* cells have the ability to carry out malate dismutation and ethanolic fermentation (Holt & Riddle, 2003). This ability has also been found in other organisms like parasitic nematodes; these parasites make use of a dedicated mitochondrial electron transport chain to achieve the purpose (Becaria *et al.,* 2002). This transport chain is not composed of oxygen, which is usually the case but, is made of fumarate. The fumarate serves the purpose of an electron acceptor, and the waste products that are produced include succinate, propionate and acetate; compared to the waste products when the chain is made of oxygen, which are carbon dioxide and water (Hulme & Whitesides, 2011).

The *C. elegans* have quite a reservoir of fatty acids, as has been described earlier, and these fatty acids enter the body by means of the bacteria that they feed on; it can also be obtained by *de novo* biosynthesis. In *C. elegans* the main proportion of the fat lies in the gut but it can also be stored in the form of drops in intestinal cells and also in the hypodermis (Kimura *et al.,* 1997). *C. elegans* have the ability similar to humans to produce and to consume the branched chain, saturated, polyunsaturated and monounsaturated fatty acids. *C. elegans* also have the ability and the required molecular composition to produce ubiquinone, farnesyl pyro-phosphate, sterols, polyisoprenoid molecule and a precursor of dolichols (Kurzchalia & Ward, 2003). This production process is conserved for both mammals and nematodes However, the nematodes lack the ability to generate sterols *de novo* using farnesyl pyrophosphate. Therefore, it is necessary that the diet of the nematode comprises of processed sterols (Hulme & Whitesides, 2011).

Lowering the level of cholesterol in humans is of great interest and chemicals and techniques have been developed for achieving this purpose. It has been discovered that a class of drug named ‘statins’ helps to lower the cholesterol level by inhibiting the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (this plays an important role in biosynthesis of sterols by farnesyl pyrophosphate). The media that is generally preferred for *C. elegans* in the labs is cholesterol. Therefore, nematodes can be useful in investigating the statin drug for its effects, benefits and harms (Mörck *et al.,* 2009). Besides having processed sterols, nematodes must also have their diet supplemented with heme and specific amino acids. Details regarding essential amino acids for both humans and nematodes have been listed by (Hulme & Whitesides, 2011). It has been observed from analysis of the genome of *C. elegans* that it contains the sequences of a number of proteins that contain heme, including cytochrome P450s, guanylatecyclases, catalases, adenylatecyclases, globins, and cytochromes that are used in cellular respiration (Rao *et al.,* 2005). Bacteria and yeast are found to lack the enzymes that synthesise heme.

However, if bacteria are used as a dietary component for *C. elegans*, then the bacteria can aid in the process of heme production (Braeckman *et al.,* 2009). Other methods of inserting heme into the nematodes include the addition of heme-containing proteins or hemin chloride, like myoglobin, cytochrome c or haemoglobin, in the medium (Braeckman *et al.* 2009).

1.10 TOXICOLOGICAL STUDIES USING *CAENORHABDITIS ELEGANS*

Metals are a part of the environment and can be easily found in the Earth's crust as well as concentrated in a number of other anthropogenic sources like waste products from factories, industries and chemical products. These metals bring about significant damage to the human body. The constant exposure to high metal concentration not only harms the human but all living organisms. With increased human activity, exposure to metal has increased considerably, and thus, the issues related to it have also increased. Metals can enter the body through contamination of food, water or the air that is inhaled and cause toxicity and other hazardous health effects including neurodegeneration and carcinogenesis. The seriousness of the effect is determined by the concentration level and the duration for which the metal remains in contact with the body. Both essential and non-essential metals are harmful for the body: Arsenic, Manganese, Silver, Uranium, Mercury, Cadmium, Antimony and Copper have all been reported to cause toxicity (Rainbow & Luoma, 2011). At the molecular level, metals affect enzyme activity, disrupt protein binding and normal cellular operation (including cell cycle progression, apoptosis, and proliferation), impairing DNA repair and disturbing homeostasis by the

production of oxidative stress and inhibiting enzyme activity (Beyersmann & Hartwig, 2008).

The process of removing or reducing metal content from the body is very difficult because of the complex human structure. But still great research has been carried out for finding out ways to solve the metal issue. The techniques that have been developed so far are based on either of the following basic concept for removal of metal and curing the body: (1) activating the organism's general stress response mechanisms (2), decreasing the uptake of the metal (3), stimulating the expulsion of the metal (4), Also chelation therapy is used to treat metal poisoning.

The organism of focus in this study, because it has ~60%–80% of the same genes as humans (Kaletta & Hengartner, 2006) and also similar regulatory proteins (Leung *et al.*, 2008), provides a laboratory model that is easy to manipulate and understand and the techniques lack the complexity when compared to the methods required for studying mammals. *C. elegans* are small in size (~1.5 mm adult), have a fast lifecycle (~3 days) and a short lifespan (~3 weeks). They are also well defined physiologically and developmentally at all molecular and genetic stages, and because of these numerous advantages, this organism has been utilized in a number of toxicity studies (Leung *et al.*, 2008). The structure of this nematode is very simple and the entire body comprises of approximately 1000 cells; thus attracting the researchers for opting this organism as a model system. A *C. elegans* hermaphrodite produces almost 300 offspring, out of which approximately 1% are male, thus allowing to get utilized for experiments (Brenner, 1974).

Brenner (1974), carried out a study on methods for the isolation, complementation and mapping of mutants, which resulted in the selection of *Caenorhabditis elegans* as model system for genetic research. Since then it has become a well establish model for toxicological tests (Jonker *et al.*, 2004; Williams, 1999; Donkin & Dusenbery, 1994;

Tatara, 1998). Williams & Dusenbery (1988), made use of *C.elegans* for testing the hazardous effects of metals such as Mercury (Hg), Beryllium (Be), Zinc (Zn), Strontium (Sr), Copper (Cu), Cadmium (Cd), Lead (Pb) and Aluminium (Al) in mammals Figure 9. The tests were validated by the fact that data obtained from the tests were similar in many respects to the mammalian data. Williams (1990) further carried a study by placing the nematodes in culture dishes and then using a buffer solution containing different dissolved metals which then came in to contact with the nematode Figure 10.

The parameters that are observed for toxicity in the *C. elegans* nematode include lifespan, reproduction, and mortality. Other parameters and observations that can be made include head thrashing, locomotive behaviour and body bending which are noted through behavioural endpoints (Williams & Dusenbery, 1988). It has been discovered that cellular systems like Heat Shock Proteins (HSPs), Glutathione (GSH), Metallothioneins (MTs) and different pumps and transporters help in removal of metals from the body (Martinez-Finley & Aschner, 2011).

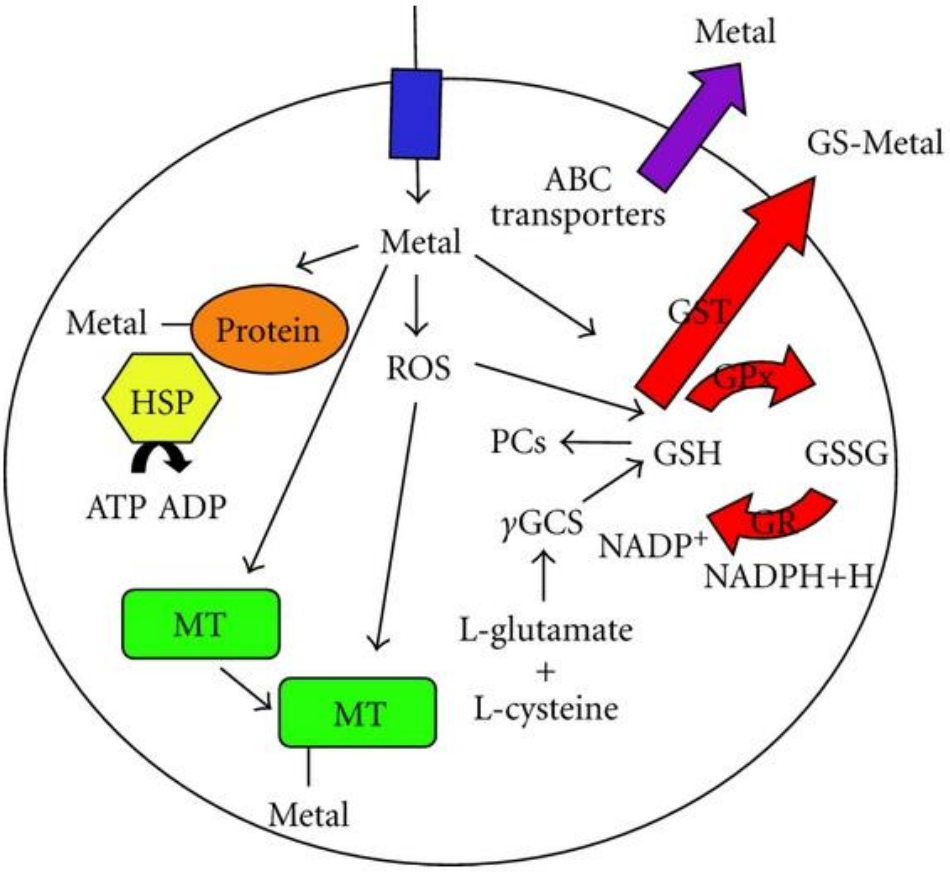

Figure 9: Diagram represents the metal detoxification system. It shows that when Reactive Oxygen Species (ROS) is exposed on the Glutathione (GSH), it converts to Glutathione disulphide (GSSG). On the other hand, the GSSG gets converted back to GSH by Glutathione reductase (GR) and it also helps the conversion of the Nicotinamide adenine dinucleotide phosphate (NADPH to $NADP^+$). In this process of synthesizing GSH, γ-glutamyl cysteine synthetase (γGCS) acts as the rate-limiting enzyme. GSH binds free radical to metal generated ROS. Metallothionein (MTs) act as antioxidants because it binds and isolates the toxins metals such as Copper, cadmium etc. Phytochelatin (PCs) behave as chelators by combining with metals. Isolated metals and those in conjugation with GS- can be excreted by the help of the ATP binding cassette (ABC) transporters. The ATPs play a major role is isolating and deactivating the metals by getting converted into ADP. This conversion is achieved by using the ATP binding proteins known as HSP70s (The 70 kilo Dalton heat shock proteins). Figure taken from (Martinez-Finley & Aschner, 2011).

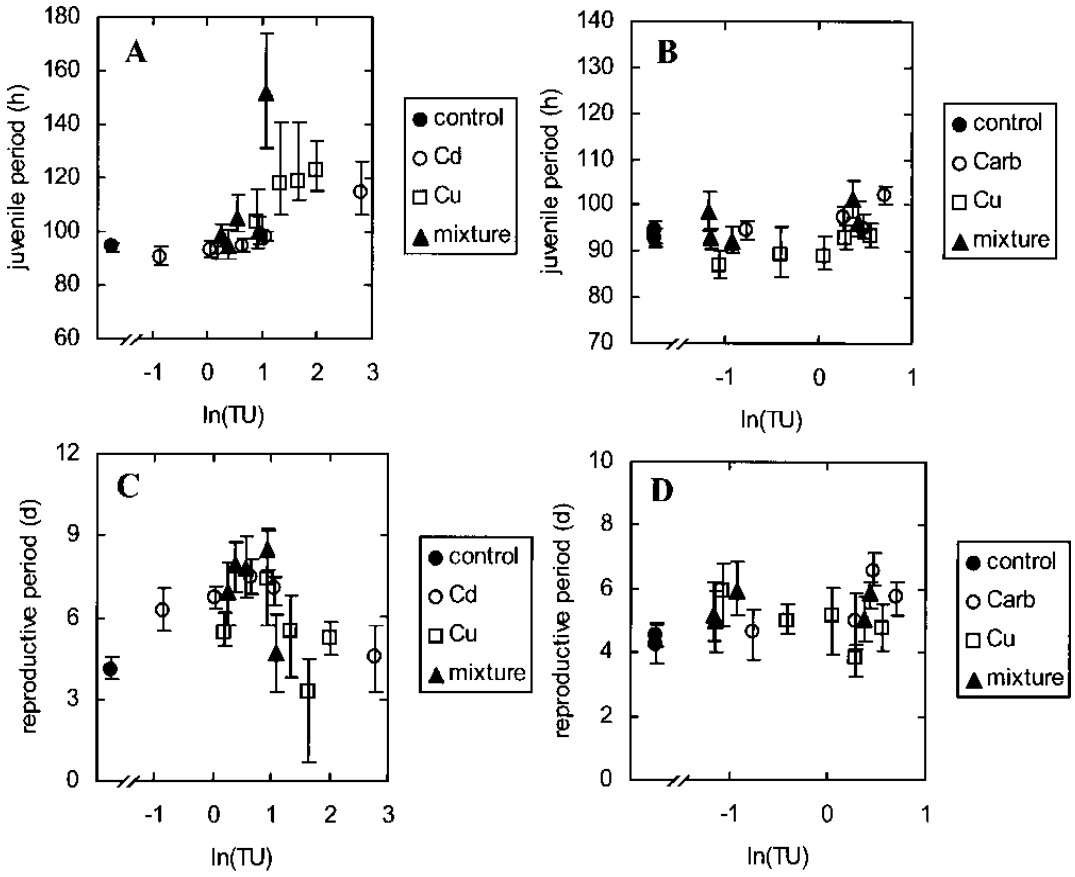

Figure 10: The effect of Carbendazim-Cu and Cadmium-Cu. The length of the reproductive period (C) and also the length (h) of the juvenile period (A and B) have been depicted. The upper black dot in (B) and lower black dot in (D) represents the ethanol control. The effective concentration of reproduction was observed on day 5, and accordingly, the concentrations were recalculated to toxic units (TU). Chart taken from (Jonker *et al.*, 2004).

Zinc is an important constituent of the body as it aids in a number of activities and processes in the body; but in excess, it can be harmful and *C. elegans* have been proved to be a useful model to study these effects. It was observed that if these nematodes were exposed to even 0.2 mM of Zinc, the development of the nematodes became 0% that in the absence of Zinc growth was 100%. It was also found out that the reproduction process considerably slowed down in the presence of Zinc and when the Zinc concentration was further increased the reproduction stopped (Bruinsma *et al.*, 2008, Chu *et al.*, 2002) Figure 11.

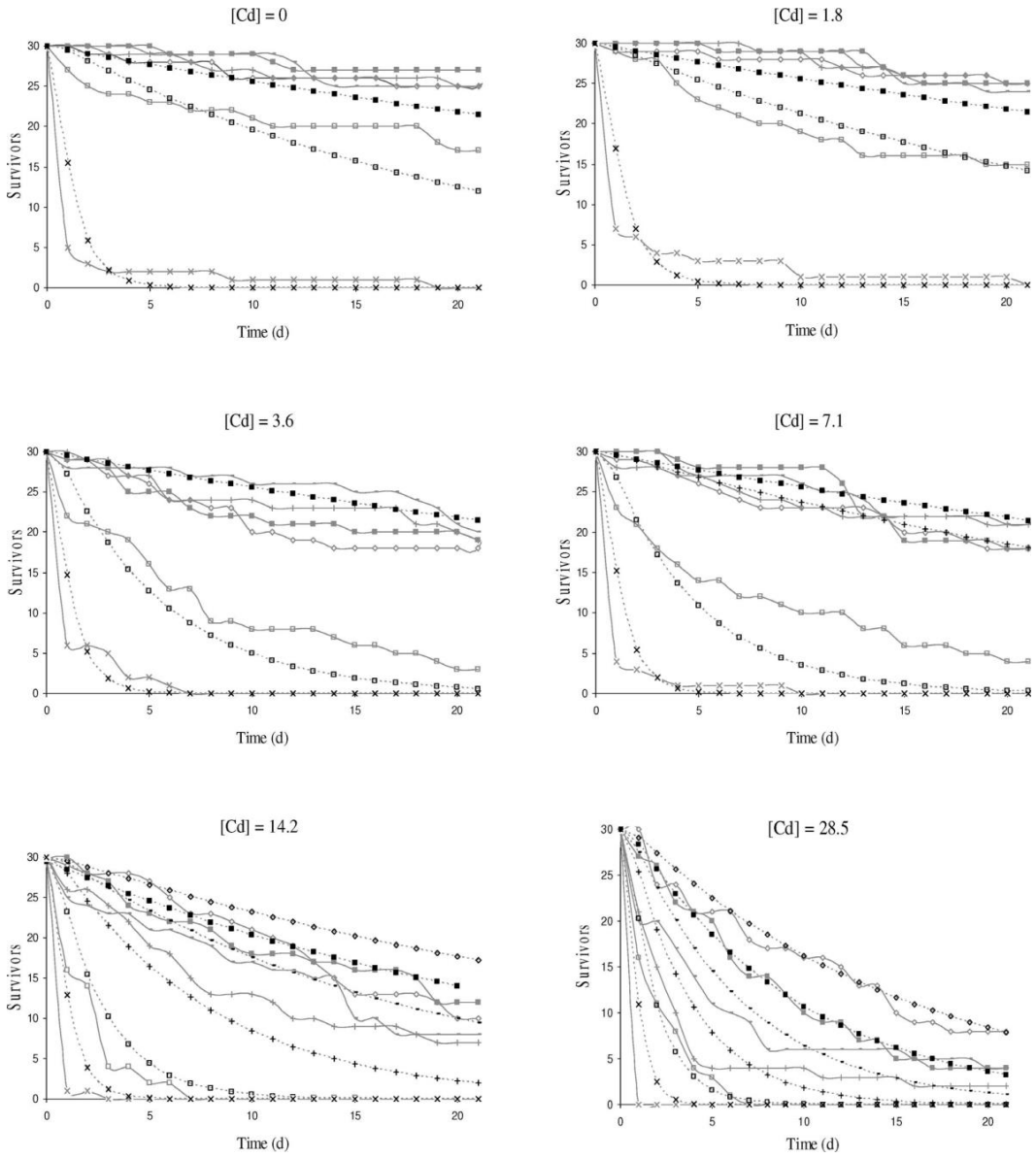

Figure 11: A comparison of the observed data and the model values. Solid lines represent the observed data while the dashed lines represent the model data. It is observed that the Copper concentration increases whereas the cadmium concentration is constant. The symbols ◊, ■, □, -, +, and × depict Copper concentrations of 0, 6.3, 12.6, 25.2, 50.4, and 101 µmol/g dry soil, respectively (Baas *et al.*, 2007).

Besides Zinc, several other metals have also been tested using *C. elegans*. Disease (2008) shows the harmful effects of Copper. The harmful health effects of Aluminium include damage to the reproduction system, brood size, body size, etc. Likewise, the increment in Al concentration led to the increase in generation time of the nematode. The rate of hatching offspring also decreased when the nematode was present in an environment consisting of $AlCl_2$ Figure 12.

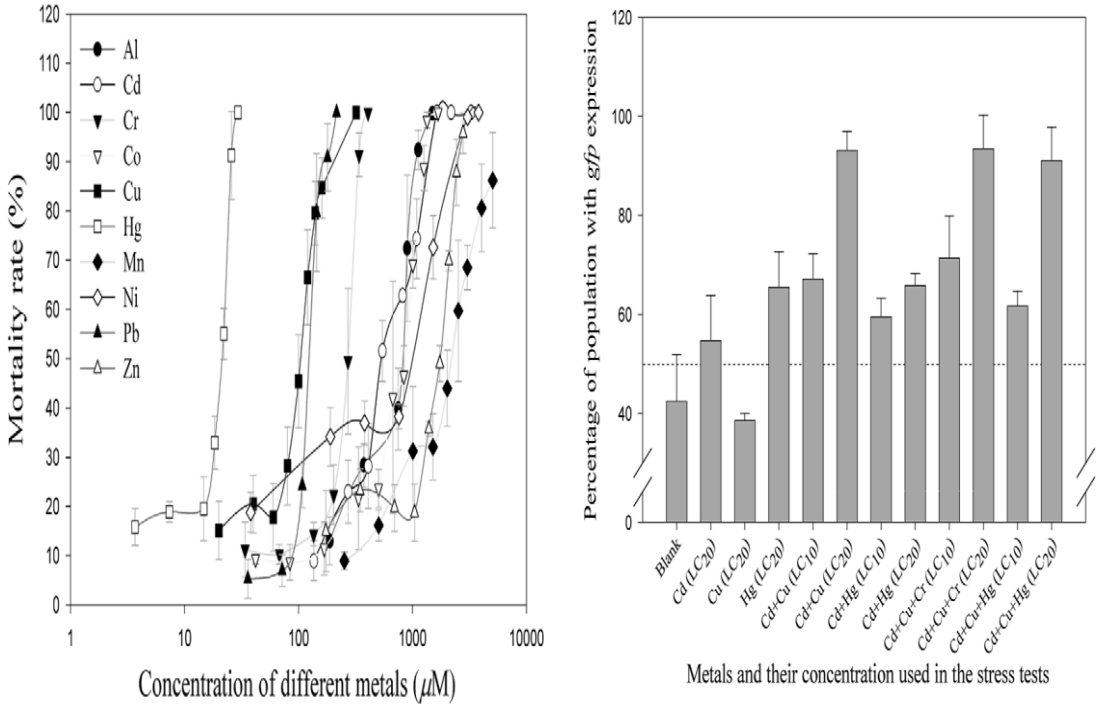

Figure 12: Dose response curve of different metals. The mortality of L1 nematodes is plotted against the testing concentration on a log scale. (A) The death rate of nematode have been plotted against concentration of metals using a log scale depicting the dose response curves of different metals. (B) The stress analysis shows the interactions between the heavy metals. Figure adapted from (Chu *et al.*, 2002).

1.11 *CAENORHABDITIS ELEGANS* ENDPOINTS

C. elegans has been found to be an ideal model test organism because the toxicity for a number of different parameters can be easily tested. Lethality and effect on other sub-lethal endpoints like growth, reproduction and feeding have been studied. The lethality endpoints are determined by comparing the number of dead organisms and the living organisms after metal exposure. Many other parameters have been observed and discussed in detail by a number of researchers (Williams & Dusenbery, 1988; Williams, 1990; Khanna *et al.*, 1997; Tatara, 1998; Donkin & Dusenbery, 1994; Hitchcock *et al.*, 1997; Dhawan & Dusenbery, 2000).

A study on the endpoints of development and reproduction was carried out on the larva of nematodes and their growth to adulthood was observed in the presence of metals (Donkin & Dusenbery, 1994). The results were obtained by observing the number of offspring that could grow into healthy adults. On the other hand, other researchers obtained results by counting the brood number or by comparing the number of eggs and the viable offspring (Whm *et al.,* 1989; Middendorf & Dusenbery, 1993; Dhawan *et al.*, 1999, Anderson *et al.,* 2001). Assessing can also be done by counting the number of eggs laid per nematode (Baldwin *et al.,* 2011).

1.12 STUDY AIMS AND OBJECTIVES

The coastal marine environment of the Red Sea is subject to direct and indirect influences of major populations and industrial facilities found along the coast such as those proximal to Saudi Arabia's second largest municipality, Jeddah City. To evaluate the ecological impact of the diffuse pollution associated with this urban conurbation, it was important to consider the interactions between contaminates that individually are below recognised toxicological thresholds but together may result in significant environmental consequences. In order to determine what mixture effects need to be considered, it was essential to define the geochemical parameters by performing a survey for major classes of contaminants and to evaluate their penetrance into the food chain. Sampling of both sediment and sea water was performed at contrasting sites representative of near-shore (25 m) with off-shore (10 km) locations. Possible food-chain transference was evaluated by sampling fish (*L. nebulosus*) and plankton at the off-shore sites. All samples were assessed for the presence of heavy metals by (ICP-MS) and hydrocarbons by (GC-MS).

Geochemistry results in specific Red Sea sampling sites in this study achieved our objective of defining the contaminant profiles within the water column and transference to the biota. Naturally, a wide range of chemicals are found concurrently in the environment, which makes identifying the contribution of individual toxicants difficult. *Caenorhabditis elegans* was deployed as a laboratory model to evaluate the combinatorial toxicology of three metal ions, Copper, Zinc and Aluminium, which were identified in the Jeddah City survey and exhibit contrasting chemistries. Toxicity tests were designed to initially determine the impact of single metal ions prior to determining mixture effects through paired metal exposures.

For the purposes of this study, the toxicity of paired metals on the nematode *Caenorhabditis elegans* was investigated. Toxicity tests were designed to first determine the impact of single metal exposure Copper, Zinc and Aluminium to different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}). Then nematodes were exposed to paired combinations of Copper, Zinc and Aluminium for 24 and 72 hours to different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) of each metal. Exposures with paired metals revealed a variety of interactions that ranged from antagonistic to synergistic effects.

The major objectives of this study were: 1) To re-evaluate the inorganic and hydrocarbon inputs into the marine environment in the vicinity of the study area; 2) To assess the major environmental contaminants both singularly; and 3) To retest each single metal in low doses with secondary metal to identify interactions that may lead to synergy or additive effects. This project has provided with both insights into mechanistic molecular toxicology, novel experimental approaches to characterize chemical interactions and the translation of laboratory investigations into developing tools for environmental monitoring and risk assessment.

2 MATERIALS AND METHODS

2.1 MATERIALS, REAGENTS AND SOLUTIONS

A list of consumables and the suppliers are detailed in Table 1 while the sources of reagents are provided in Table 2. Specific solutions and buffers that prepared according to the compositions shown in Table 3. For amino acids standards components, it is been detailed in Table 4.

Table 1: A list of suppliers of consumables.

Supplier	Consumables
Fisher Scientific, UK	Disposable plastic-ware.
Thistle Scientific, UK	1.0 mm zirconia/silica beads. 2.0 ml tubes with screw caps, clear, pk 500.
Fisher Scientific, UK	Autoclave bags, printed biohazard 40 um thickness opening width 620 x 720.
VWR International Limited	6-well micro-titre plates, tissue culture treated, flat bottomed with lid. 24-well micro-titre plates, Nunclon Surface, flat bottomed with lid.
StarLabs, UK	3 ml micro-centrifuge tubes. Pipette tips of different size.
Eppendorf, UK	1.5 ml Eppendorf tubes.
Sigma Chemical Company, UK	Acid washed glass beads. 2 ml vials clear glass with screw top cap.
Greiner Bio-One, USA	90 mm diameter petri dishes.

Alpha Laboratories, UK	2 ml Cryogenic vials.
BD, UK	15 ml Falcon™ conical polystyrene tubes. 50 ml Falcon™ conical polystyrene tubes.
Phenomenex, UK	4 * 96 Sorbent tips in racks. 4 * 100 Sample preparation vials. 20-100 µl Micro dispenser. 0.6 ml Syringe. 1.5 ml Syringe. 1 ZB-AAA 10 m x 0.25 mm Amino acid analysis GC column. 4 * 100 Auto sampler vials with inserts. 5 FocusLiners™.

Table 2: Table of reagents and their suppliers.

Supplier	Reagents
BD, UK	Bactoagar, Bactopeptone. Agar, technical grade (Difco Brand) 500 g
Sigma Chemical Company, UK	Nitric acid 70%, amino acid standard, methoxyamine hydrochloride, pyridine, MSTFA, Methanol, Sodium hypochlorite solution, Nervonic acid, Cholesterol 94%, Chromium (II) chloride 95%, Cadmium chloride anhydrous, Copper (II) sulphate, Manganese (II) chloride beads 98%, Lead (II) nitrate 99.9%, Zinc chloride, Aluminium

	chloride anhydrous 99%, Supelco 37 component FAME mix (varied concentrations in dichloromethane).
Phenomenex, UK	EZ:faast GC-MS Physiological Amino Acid Analysis Kit.
NU-CHECK-PREP, USA	GLC Reference Standard 411.
LGC Standards, UK	Tuna fish tissue - Arsenic species.
Phenomenex Ltd, Deutschland	EZ:faast easy-fast amino acid sample testing kit for GC-MS.

Table 3: List of solutions, buffers and their compositions.

Method	Solution	Components
Agar Plates	NGM Agar	0.032 M KCl, 0.051 M NaCl, 2.5% Bactopeptone, 0.17% Bacto-agar, 0.01% cholesterol, 0.1 M $CaCl_2$, and 0.1 M $MgSO_4$
	Cholesterol	1% (w/v) dissolved in Ethanol.
Egg preparation	Bleaching solution	5% NaOH (10 M), 20% NaOCl.
	M9 buffer	3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl and 1 litre H_2O Autoclave 30 min then add 1ml 1M $MgSO_4$

Nematode Freezing Solution	Freezing solution	100 mM NaCl, 50 mM KPO_4 (pH 6), 30% glycerol. Autoclave and add 0.3 mM sterile $MgSO_4$.
Liquid Media	K-medium	0.032 M KCl, 0.051 M NaCl.

Table 4: List of EZ:faast easy-fast Amino Acid sample testing kit reagents

Reagent	Ingredient	Volume
Reagent 1 (Internal standard solution)	Norvaline 0.2 mM N-propanol 10%	50 ml
Reagent 2 (Washing solution)	N-propanol	90 ml
Reagent 3A (Eluting medium component I)	Sodium hydroxide	60 ml
Reagent 3B (Eluting medium component II)	N-propanol	40 ml
Regent 4 (Organic solution I)	Chloroform	4 vials, 6 ml each
Reagent 5 (Organic solution II)	Iso-octane	50 ml
Reagent 6 (Re-dissolution solvent)	Iso-octane 80% Chloroform 20%	50 ml

2.2 PREPARATION OF EQUIPMENTS

All pipette tips and micro centrifuge tubes were autoclaved at 121°C and around 15 psi for 20 minutes. Nematode picks were dipped in 100% ethanol prior to use. For nitric acid digestion, glassware was acid washed. Glassware was placed in a solution of 10% sodium dodecyl sulphate and scrubbed. Following several distilled water rinses the glassware was placed in 7.0% nitric acid solution and scrubbed. Then the glass was dried overnight.

2.3 RED SEA GEOCHEMISTRY TEST

2.3.1 STUDY AREA AND COLLECTED SAMPLES

The current work was conducted in the central part of the eastern Red Sea coast with eight sampling areas, distributed over about 56 km stretch of Jeddah Red Sea coastal. Sea water, plankton, sediment and fish in Jeddah city area are collected in this study and each area was studied to determine heavy metals and hydrocarbons of each type of sample. All fish samples have been caught by the hook at night under fisherman expertise supervision.

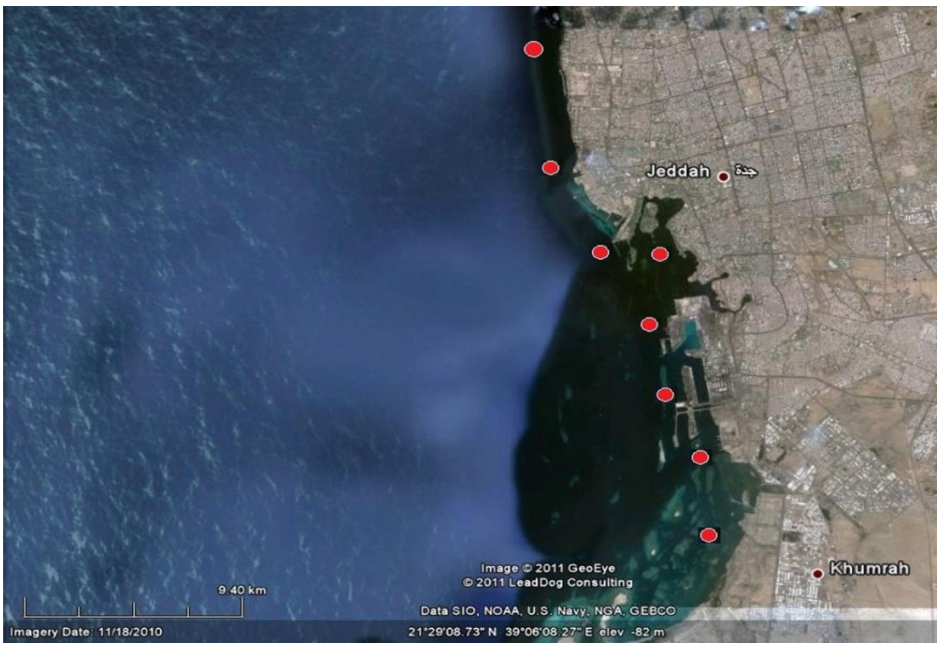

Figure 13: Study area (Jeddah city) and chosen samples locations. Red circles indicate the approximate sampling locations used for near-shore (shallow) sampling and indicate where sediment, plankton and fish were collected. Deep sea water samples were collected from sites ~10 km perpendicular from shore at the same locations.

2.3.2 SAMPLING METHOD

Five of spangled emperor *Lethrinus nebulosus* fish per each area were collected freshly, samples were immediately dissected, gills and liver of each examined fish were rapidly excised and divided into two parts and stored in ice-box until reaching the lab then stored at -80ºC till processed for the analysis. Two litres of sea water of each area was collected and transferred to the lab and kept overnight at room temperature. For sea water, one litre was collected and divided into two brown glasses without filtration and stored at 4ºC till the analysis.

2.3.3 SAMPLE PREPARATION

To minimize contamination, all materials and glassware used in the experiments were cleaned by soaking overnight in a 10% (w/v) nitric acid solution then rinsed with

ultra-pure deionized water and a stainless steel knife and scissor were used to cut the tissue. Before analysis the tissues (gill and liver) were thawed and weighted (Al-Bader 2008). For heavy metals analysis, microwave method was applied for the digestion procedure of samples. Thawed weighted samples were placed in a Teflon digestion vessel with 5 ml concentrated nitric acid (HNO_3) and 1 ml of Hydrogen peroxide (H_2O_2). The samples in the vessels were then digested using an optimized method. Then, they cooled at room temperature. The residues were then dissolved and diluted to 10 ml. The chemicals used were of analytical grade (Uysal *et al.*, 2008).

Plankton samples were collected from two litres of sea water that was filtered using a pre-weighed 0.45 µm Millipore membrane filters. The filters were dried at 80ºC to constant weight. Then dry filters were reweighted, to get the plankton weight, and digested by concentrated HNO_3 at 80ºC the residues were dissolved and diluted to 2 ml according to method described previously (Riley & Segar, 1970).

For hydrocarbons determination, freeze dried fish tissue was weighted into 50 ml amber glass bottle and the internal recovery standard solution was added. Then, a mixture of Chloroform/Methylene chloride (CH_3OH/CH_2Cl_2) (2:3) was added and the sample shaken vigorously. This was achieved pouring the solvent into the separation funnel fitted with fritted glass and the extract was decanted into pear-shaped evaporating flask. The CH_3OH/CH_2Cl_2 (2:3) mixture was then added to separation funnel and subsequently shaken after which the extracted was decanted into evaporating flask. Finally, the combined extract was stored at -80ºC until analysis (Almohanna, 2000).

2.3.4 HEAVY METALS ANALYSIS

Al, Cr, Fe, Mn, Co, Ni, Cu, Zn, As, Se, Ag, Cd, Sn and Pb in fish and plankton were determined by a Perkin-Elmer inductively coupled plasma-optical emission

spectrometry (ICP-OES) in Saudi Geological Survey (SGS). In the ICP-OES analysis, the wavelength lines were used: Copper 324.8 nm, Zinc 213.9 nm, Manganese 232 nm, Nickle 324.7 nm, Chromium 357.9 nm, Cobalt 240.7 nm and Lead 283.3 nm. The quality of the analytical process was also controlled by the analysis of NIST-CE278 certified standard material (Uysal *et al.*, 2008). For sea water samples, heavy metals were determined by using inductively coupled plasma mass spectroscopy (Perkin-Elmer ICP-MS) in Saudi Geological Survey (SGS).

2.3.5 HYDROCARBONS ANALYSIS

Hydrocarbons in fish and plankton samples were determined by an Agilent gas-chromatography mass spectrum (GC-MS). For sea water hydrocarbons were determined by a Thermo Gas-Chromatography mass spectrum with purge and trap system. The gas chromatography configuration used were: Initial temp: 260ºC, interface temp: 230ºC, control mode: split, column inlet pressure: 100 kPa, column flow: 1.6 ml/min, split ratio: 21, oven temp: 60ºC keep for 1 min to 280ºC keep for 31 min and at a rate of 10. For mass spectroscopy parameters used included: Acquisition mode: scan, interface temp: 230ºC, solvent cut time: 3 min, detector gain mode: absolute and detector gain: 1.30 kV.

2.4 *C. ELEGANS* TOXICITY TEST

2.4.1 *E. COLI* CULTURES

E. coli OP50 cells were grown up in LB broth medium. LB agar was prepared and poured under sterile conditions to make LB agar plates. These plates were then streaked with a single colony of OP50 *E. coli* and incubated at 37ºC overnight. (Only one plate was streaked at a time, the remaining LB agar plates were sealed and stored at 4ºC). A

single colony from this plate was used to inoculate of LB broth in a universal (50 ml) tube under sterile conditions. This was then grown overnight at 37ºC whilst being agitated by shaking at 240 rpm. OP50 *E. coli* plates and liquid media were stored at 4°C. A new LB agar plate was streaked with OP50 *E. coli* every 4 weeks.

2.4.2 NEMATODE CULTURE

Wild type strain N_2 *Caenorhabditis elegans* was used in this experiment (Girard *et al.,* 2007). The dauer larval stage was kept as a stock in M9 buffer at 20ºC and was renewed monthly. Age synchronous adult nematode were generated by using the dauer for toxicity tests. The dauers were placed on NGM agar plates (0.032 M KCl, 0.051 M NaCl, 2.5% bacto-peptone, 0.17% bacto-agar, 0.01% cholesterol, 0.1 M $CaCl_2$, and 0.1 M $MgSO_4$) with an established lawn of OP50 (uracil-deficient strain of *Escherichia coli*) for a food source and were maintained at 20ºC. After 3 days, eggs were collected and placed on NGM agar plates inoculated with a viable OP50 lawn and were subsequently maintained for 3 days to obtain age-synchronous adults (Anderson *et al.,* 2001).

2.4.3 MAINTENANCE OF *C. ELEGANS* STRAINS

Nematodes stocks were maintained on Nematode Growth Medium (NGM) agar in a constant temperature incubator at 20ºC. To sustain *C. elegans* population, a large number of nematodes were transferred to new plates by chunking weekly (Girard *et al.*, 2007). Smaller numbers and individual nematodes were transferred between plates using a nematode pick was made from a horse hair attached to a small stick.

2.4.4 EGG PREPARATION

Egg preparation was performed to age-synchronise nematodes at the L1 stage. Gravid adult were washed off agar plates with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl and 1 litre H_2O which was autoclaved for 30 min prior to the addition of 1 ml of sterile 1 M $MgSO_4$) into Eppendorf tubes. Nematodes were then centrifuged at 12,000 g for 2 minutes after which the supernatant was discarded and nematode pellet recovered. Bleaching solution (5% NaOH (10 M), 20% NaOCl) was then added to the pellet and vigorously shaken for 3 minutes. The tubes were then centrifuged at 12,000 g for 1 minute and supernatant removed. The pellet was then washed with M9 buffer prior to re-centrifugation for 1 minute to allow the supernatant to be removed. This washing step was repeated three times. M9 buffer (1 ml) was added to the resultant pellet and the tubes kept overnight at room temperature which allowed eggs to hatch and suspended their development at the L1 stage. Then L1 nematodes were then centrifuged gently at 2,500 g for 2 minutes before being transferred to a fresh new agar plate (Girard *et al.*, 2007).

2.4.5 FREEZING NEMATODENEMATODES

C. elegans can be frozen and stored at -80ºC for an indefinite period of time. Starved L1 nematodes are most likely to survive this freezing. In order to obtain starved animals nematode to be frozen, nematode populations are either bleached to obtain starved L1 larvae or allowed to grow on plates until the OP50 *E. coli* was exhausted. Plates containing starved *C. elegans* nematodes were washed with 2-3 ml of M9 buffer and placed into a 5 ml centrifuge tube. An equal volume of freezing solution (100 mM NaCl, 50 mM KPO_4 (pH6) with 30% glycerol sterilised by autoclaving and with subsequent addition of 0.3 mM sterile $MgSO_4$) was added and mixed by inverting several

times. Aliquots (1 ml) were then dispensed into 1.5 ml cryovials. The tubes were closed, labelled and frozen at -80ºC.

2.4.6 PREPARATION OF TEST SOLUTIONS

Metal chloride salts of Aluminium and Zinc and Cu_2SO_4 (Sigma-Aldrich Chemical, Milwaukee, WI, USA) dissolved in K-medium (0.032 M KCl, 0.051 M NaCl) were used to prepare four test concentrations with control solution being K-medium with no additional salts. Test solutions for the single metals were made by weighing, transferring to a 1 L flask to make a stock solution. Appropriate aliquots were then calculated, measured volumetrically, transferred to the test plates and diluted to volume with additional K-medium. Mixture test solutions were prepared in a similar fashion. All control solutions consisted of K-medium only (Williams, 1990).

Table 5: The lethal and the calculated sub-lethal concentrations used for the different heavy metals studied. Lethal concentration used are those reported by Chu *et al.,* 2002 *(Chu *et al.,* 2002).

Metal	LC_{10} (µM)	LC_{20} (µM)	LC_{30} (µM)	LC_{40} (µM)	LC_{50} (µM)*
Al	135	269	404	538	673*
Cu	19	37	56	75	93*
Zn	340	679	1019	1358	1698*

* LC_{50} calculated using 48 h exposures at 22ºC

2.4.7 TOXICITY TESTING

Adults were collected from plates and prepared for transfer to test wells. Nematodes were loaded into 1 ml wells of 24 well tissue culture plates. Each test well

typically contained K-medium (0.032 M KCl, 0.051 M NaCl) and inoculated with *E. coli* OP50, with or without toxicant. The initial bacterial optical density (absorbance at 600 nm) used in measurement of feeding was determined immediately after nematodes were transferred. Plates were transferred to an incubator at 20ºC for periods 72 hours (Anderson *et al.,* 2001).

2.4.8 MORTALITY

Mortality was assessed using the 72 hours tests described by (Dhawan & Dusenbery, 2000). Nematodes (n=10) nematode from age-synchronized cultures used for testing were placed in 1 ml of test solution. Three days later, at 72 hours, the number of survivals nematodes was counted. For each test concentration and control, the mean number of survivals from the wells was obtained for each trial, and at least three trials were conducted. Each trial included a control and four toxicant concentrations. All tests were carried out in the presence of viable *E. coli* OP50. Three trials were run for all of the toxicants Copper, Zinc and Aluminium using the LC_{10}-LC_{40} concentrations calculated previously and shown in Figure 5 (Chu *et al.,* 2002). Survival was measured after 24, 48 and 72 hours of exposure.

2.4.9 REPRODUCTION

Reproduction was assessed using the 72 hours tests described by (Dhawan & Dusenbery, 2000). Single nematode from age-synchronized cultures used for testing with other endpoints were placed in 1 ml of test solution. Three days later, at 72 hours, the number of offspring at all stages beyond the eggs was counted. For each test concentration

and control, the average number of progenies from the wells was obtained for each trial, and at least five trials were conducted.

2.4.10 METABOLITE EXTRACTION

Extraction strategies for comprehensive metabolome coverage in *C. elegans* were previously assessed by (Geier *et al.*, 2011). As GC−MS was intended to be use for the analysis, a methanol/chloroform extraction technique was performed to give good overall metabolomic coverage. Nematode collected from the large plates were pelleted by centrifugation (400 g) and then mixed with 600 µl of a methanol/chloroform mixture (2:1 v/v) and transferred to bed beater machine (FastPrep®-24, MP Bio) for 5 times in 5 min to extract the metabolites from nematodes body, after each 1 min nematodes were put in ice to maintain the temperature of the process. Two aliquots (200 µl) of both chloroform and water were then added, and the samples were centrifuged for a further 20 min. This resulted in the formation of aqueous and organic layers, which were transferred to separate micro-centrifuge tubes. The aqueous layer was dried overnight in a Concentrator 5301 evacuated centrifuge (Eppendorf, Histon, UK) and analyzed via GC−MS. The lipid fraction was dried overnight in air and analyzed using GC−MS. Samples after prepared were run in Cardiff University, Biosciences School, under expertise technician.

2.4.11 GC−MS OF AQUEOUS FRACTION

Using and following EZ:faast kit method (Phenomenex, UK), eluting medium was prepared in capped tubes by the addition of 3:2 parts Reagent 3A with Reagent 3B prior to thorough mixing by vortexing. Labelled sample preparation vials were placed in a tray with 100 µl sample, pH range 1.5-6.0. Reagent 1 (Manufacture's Internal Standard) (100

µl) was then added to the sample vial. The sample was then passed slowly through a sorbent tip, fitted to a 1.5 ml syringe, to allow amino acids and changed metabolites to bind to the matrix within the tip. The liquid was then expelled from the sorbent tip to waste before continuing to draw any further sample through sorbent. When all samples had been bound to the tip and liquid removed Reagent 2 (200 µl) (washing solution) was then pipetted through sorbent tip by drawing the fluid slowly through tip. Additional air pressure was required was then used to pull wash solution through sorbent bed. The tip was separated from syringe left in the sample tube. The contents of the syringe were then discarded by using the 0.6 ml syringe barrelled pulled half way up, attaching to tip and expelling air through the sorbent bed. Previously prepared eluting solution (200 µl) was pipetted into a sample vial and used to wet the sorbent particles with care not to allow the liquid to reach the filter plug. Particles and liquid were then ejected into the sample vial and this was repeated until all the particles were washed out of the tip. Using the Drummond micro Pipette, 50 µl of Reagent 4 was pipetted into the vial and the mixture vortexed until milky. The resultant solution was allow to stand for >1 min until two layers had form after which the vortex procedure was repeated and the sample allowed to stand for a further 1 min. Using Drummond micro Pipette 100 µl (2*50 µl) of Reagent 5 was transferred into the sample tube. Subsequently the sample tube was vortexed and then allowed to stand for > 1 min. A Pasteur pipette was then used to transfer the organic top layer into to GC vial avoiding any contamination from the lower aqueous layer. The vial was then evaporated under N_2 for no more than 10 min. After drying, sample was re-dissolved in <= 25 µl of Reagent 6. Finally, samples were transferred into insert with Pasteur pipette and placed into a GC vial prior to analyses.

2.4.12 GC−MS OF LIPIDS FRACTION

The lipid content of the cell pellets was analyzed via GC−MS following the method of (Atherton *et al.,* 2008). The dried organic fraction was first dissolved in 250 µl of Chloroform-Methanol (1:1 v/v). A 100 µl aliquot of a solution of 10% BF_3-Methanol (Sigma-Aldrich) was added then vials were incubated at 80°C for around 90 min. Samples were then left to cool for almost 10 min, after that 0.3 ml of H_2O and 0.6 ml of hexane were added and each vial vortex was mixed for 1 min. The aqueous layer was discarded, and the remaining organic layer was evaporated to dryness before being reconstituted in 25 µl of hexane for analysis.

2.4.13 GC−MS PARAMETERS

A Finnegan Trace GC ultra (Thermo Fisher Scientific, Hemel Hampstead, U. K.) coupled to a Finnegan Trace DSQ mass spectrometer (operated in the EI+ mode) was utilized for metabolite analysis. Helium (1.2 mL/min) was used as the carrier gas. For the aqueous metabolites, a ZB-AAA 10 M x 0.25 mm Amino Acid Analysis GC Column (Phenomenex Macclesfield, Cheshire) was employed to perform the separation. The oven temperature program initiated at 70°C and was then increased at 10°C per minute to 130°C, then by 5°C a minute to a temperature of 230°C, and then by 20°C a minute to a final temperature of 310°C, which was held for a further 5 min. The total run time was ~34 min per sample. The column eluent was introduced into an Agilent mass spectrometer, with ion source temperature = 240°C, MS Quad temperature = 180°C electron beam = 70 eV, source current = 100 µA, and injection volume = 2 µl in all cases. The detector was turned on after a solvent delay of 1.8 min, and data was collected in full scan mode using a mass range of 45−450 m/z with 3.5 scan/sec.

For the organic phase metabolites, a TR-FAME stationary phase type column (Model Number: PE 225 ELITE 225 POLAR; 30 m × 0.25 mm ID × 0.25 μm, cyanopropyl polysilphenyl-siloxane) was used with a split-less injection. The injector temperature was set to 220°C, and the GC oven temperature program was 170°C held for 3 min to reach a final temperature of 220°C, which was held for 10 min. The total run time was 30 min per sample. The column eluent was introduced into an Agilent mass spectrometer, with ion source temperature = 230°C, MS Quad temperature = 150°C electron beam = 70 eV, source current = 100 μA, and injection volume = 2 μl in all cases. The detector was turned on after a solvent delay of 1.8 min, and data was collected in full scan mode using a mass range of 35−650 m/z with 2.42 scan/sec. GC−MS chromatograms were analyzed using ChemStation™, integrating each peak individually. Identification of individual peaks was performed by comparing the full mass spectrum for each compound with those in the inbuilt National Institute of Standards and Technology (NIST™) database (2002 edition) in conjunction with retention time matching to known standards (Supelco 37 Component FAME Mix; Sigma Aldrich). Deconvolution of overlapping peaks was achieved by overlaying traces of single ions.

2.4.14 TRACE METALS DIGESTION AND ICP-MS PARAMETERS

Nematodes were freeze-dried, reweighed to obtain the dry pellet weight, and digested by heating in a hot block digester at 90ºC for approximately 1 h with concentrated nitric acid (HNO_3) and metal concentrations were quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS - Agilent 7500). Sample digests and sample extracts were analysed for Li, Be, Mg, Al, P, S, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Y, Zr, Nb, Mo, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Tb, Gd, Dy, Ho, Er, Tm, Yb, Lu, W, Tl, Pb, Bi, Th, U. The standard operating conditions

were as follows: RF power, 1,550 W; gas flow rates: coolant, 15 l min^{-1}; auxiliary, 0.8 l min^{-1}; nebulizer, 0.85 l min^{-1}; makeup gas, 0.25 l min^{-1} and collision cell gas He, 5.5 ml min^{-1}. An internal standard solution was added via a t-piece to the sample stream containing Sc, Ge, Rh, In, Te and Er, giving approximate signal sensitivity of greater 200 k cps (Watts *et al.,* 2013).

2.5 STATISTICAL ANALYSIS AND DATA INTERPRETATION

Each reported result was expressed as mean of the individual analyses. The statistical differences of mean metal levels among tissues, sea water, trace elements, amino acids and fatty acids were analysed using SPSS™, Prism 6 (GraphPad™ Software, Inc.) software's and GENE-E (Broad Institute). Data were tested for normality using an X^2 test and for homogeneity of variance with Bartlett's test. Only data that passed these tests were used for further statistical analysis. Correlation significance was determined using one-way ANOVA as appropriate, with Tukey's *post hoc* test. Tukey's multiple comparison test ($\alpha = 0.05$) was performed to determine differences between toxicants and between endpoints for a given toxicant (Page *et al.*, 2012). A two-tailed t-test for two samples with unequal variance was used to analyze the control and treated populations for each element; *p*-values of less than 0.05 were used to determine the significance.

The following specific analyses were perform:

$$\frac{\text{((Element level for exposure) - (Mean element level for control)}}{\text{(Mean element level for control))}}$$

Percentage change calculation: Ion or metabolite (element) values for treated populations were compared to the mean value for the control population to produce the percentage change from control values used to calculate the mean percentage change from control for the graphical representation of the data.

Elemental values for treated populations were compared to the mean value for the control population to produce the percentage change from control values used to calculate the mean percentage change from control for the graphical representation of the data.

New Automatic Linear Modelling: New Automatic Linear Modelling (Linear Regression) was performed in the SPSS is carried out using the Regression method, which is capable of fitting linear models and computing a variety of model fit statistics. Researchers frequently collect a data set having a large number of independent variables and each of them is a potential predictor of the dependent variable. The problem of deciding which subset(s) of the large group of potential predictors to include in a linear regression model is; therefore, very common and arguably the hardest part of regression modelling (Ratner, 2011).

Stepwise method: This approach enters or removes predictors one at a time after taking into account the marginal contribution of a predictor controlling for other variables already in the model. Multiple criteria exist for evaluating this marginal contribution: the Partial F-test, the Akaike's Information Criterion Corrected (AICC) (Hurvich & Tsai, 1989). The stepwise method has three variants: 1- forward selection, 2- backward elimination, and 3- forward stepwise (Breaux, 1968). The forward stepwise variant is probably the most popular technique, where after each variable (other than the first) is added to the set of selected predictors, the algorithm examines to see if any of the previously selected variables could be dropped without appreciably increasing the

residual sum of squares. This process continues until the stopping criteria are met (Yang, 2013).

All-possible-subsets method: Compared with the stepwise approach that economizes on computational efforts by exploring only a certain part of the model space, the all-possible subsets approach conducts a computationally intensive search of the entire model space by considering all possible regression models from the group of potential predictors. Given p predictors, there are a total of 2^P regression models (including the intercept-only model) to be estimated. Clearly, the number of models under the all-possible-subsets approach increases rapidly as the number of candidate predictors increases. Given that the approach is computationally intensive, it works better when the number of potential predictors is not too large, for example 20 or fewer (Miller, 2002; Yan, 2009). In SPSS Statistics, the LINEAR procedure provides both the all-possible-subsets and the stepwise capability (forward stepwise only). Both approaches are guided by multiple optimality statistics. Specifically, the two variable selection platforms share three optimality criteria (i.e., AICC, adjusted R-square, and Over-fit prevention criterion) and the stepwise approach employs an additional criterion in the form of an F statistic (Fox, 1997; Fox & Monette, 1992).

3 ASSESSMENT OF HEAVY METALS CONTAMINATION IN THE RED SEA IN THE PROXIMITY OF JEDDAH CITY

3.1 INTRODUCTION

The increase in anthropogenic activities, urban combinations and industrial activities, along shorelines have resulted in serious environmental pollution mainly associated with the release of large quantities of metals ions into coastal waters (Jytte Molin, 1995). Jeddah southern corniche area receives equivalent of 300,000 m^3 of semi-treated sewage. Before 2001, the sewage was directly dumped from an outlet set at about 1 m above the sea surface. Since 2001, the same volume of civic wastewater is disposed from underwater diffuser sited at about 3 km south of the old effluent (Al-Farawati, 2010).

Previous investigations have showed that despite the very noticeable dilution effect, the impact of the effluent is measurable and extends *in situ*, north and south along the coast from the point of release (PERSGA, 2003). This has led to sediments becoming enriched in heavy metals such as Aluminium, Arsenic, Manganese, Copper, Lead and Nickle (PERSGA, 2003). Extended temporal studies have confirmed heavy metal contamination in the Red Sea water especially in the shallow water reflection human activities, in 1994 Massoud & Fahmy and in 2006 El-Ghazaly & Abdel-Aziz recorded heavy metals pollution associated with elevated levels of Manganese, Copper, Zinc and Cadmium. In order to gain an up-to-date overview of the marine environment surrounding Jeddah City and the current impact of anthropological pollution, samples of sea water, sediment, plankton and fish were collected and the levels of heavy metals and hydrocarbons determined. To investigate how metals levels were impacted by local currents and tides associated with the Jeddah City locality, shallow (near-shore) and off-shore water samples were acquired and analysed.

3.2 RESULTS

3.2.1 SAMPLING

To study the extent to which costal pollution impacted on the wider Red Sea ecosystems, sea water samples were collected from both near-shore (25 m from shore) shallow sampling areas, 5 m depth, and from off-shore (10 km from shore) locations, 65 m depth, taken perpendicular to the coast approximately west of near-shore locations Figure 14. Sampling sites were selected on those previously studied (Massoud & Fahmy, 1994; Montaser *et al.,* 2010). Furthermore, tropic transfer was investigated by determining the metal levels in sea water, sediment, plankton and wild fish populations caught at the off-shore sampling stations. Triplicate independent samples were gathered for each sample type at each station and these were transferred to the laboratory for analyses. No significant differences were identified between sites, and therefore, the results presented represent an average of the data acquired from all samples.

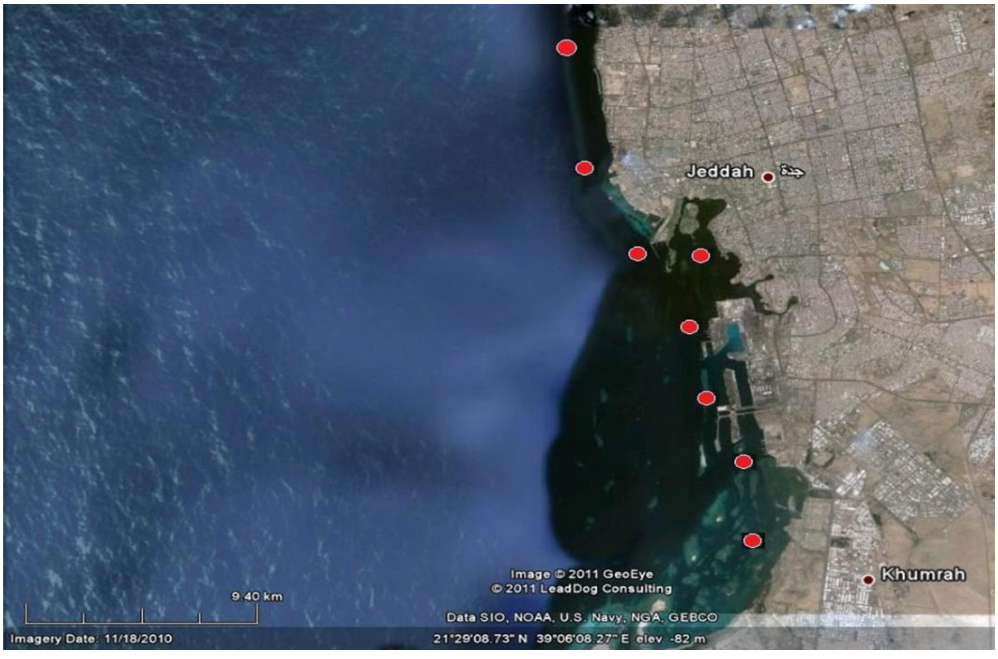

Figure 14: Jeddah City sampling locations. Red circles indicate the approximate sampling locations used for near-shore (shallow) sampling and indicate where sediment, plankton and fish were collected. Deep sea water samples were collected from sites ~10 km perpendicular from shore at the same locations.

3.2.2 HEAVY METAL ANALYSIS OF SEA WATER

Heavy metals are measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (see Section 2.4.14). Analysis of the metal composition of the water samples collected at the off-shore sampling stations Figure 15 indicated that Copper was the highest represented metal ion with an average concentration of 57.76 µg L^{-1}, followed by Arsenic at 43.00 µg L^{-1} and Nickle at 32.00 µg L^{-1}. Lead was the least abundant metal at 0.79 µg L^{-1}.

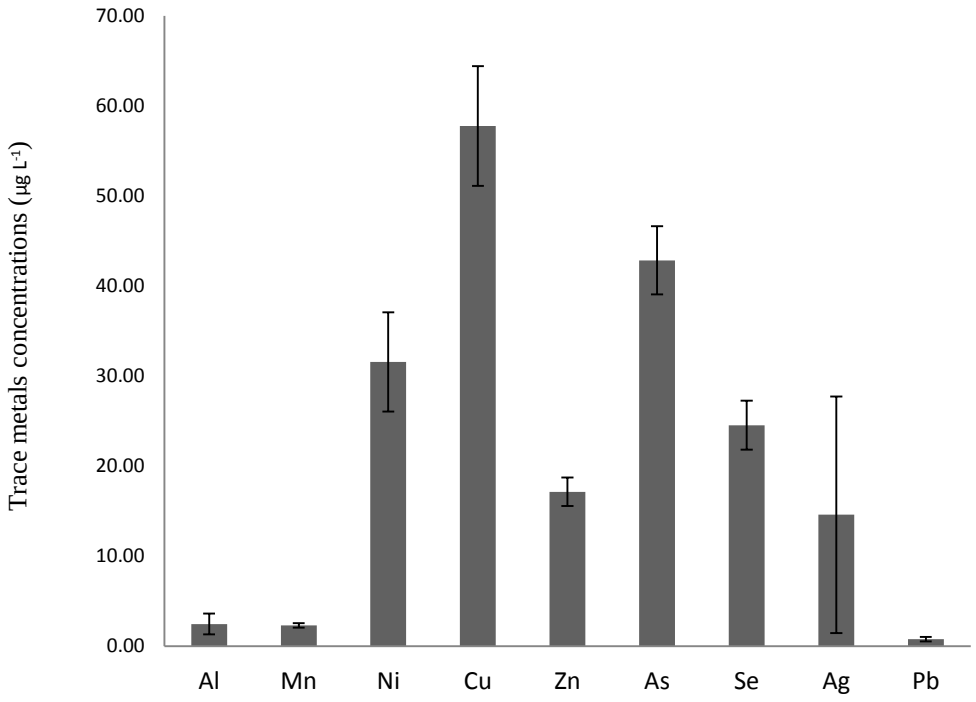

Figure 15: Heavy metals concentrations of Jeddah Red Sea at off-shore sampling stations. The study area encompassed the central part of the eastern Red Sea coast with eight sampling areas distributed over about 56 km stretch of the Jeddah Red Sea coastal samples were collected near the sea surface and resulted in µg L^{-1} as measured by ICP-MS.

In contrast for heavy metal concentrations in the near shore samples Lead was the most abundant metal at 97.00 µg L^{-1}, followed by Arsenic and Nickle represented at 47.00-32.00 µg L^{-1}, respectively Figure 16.

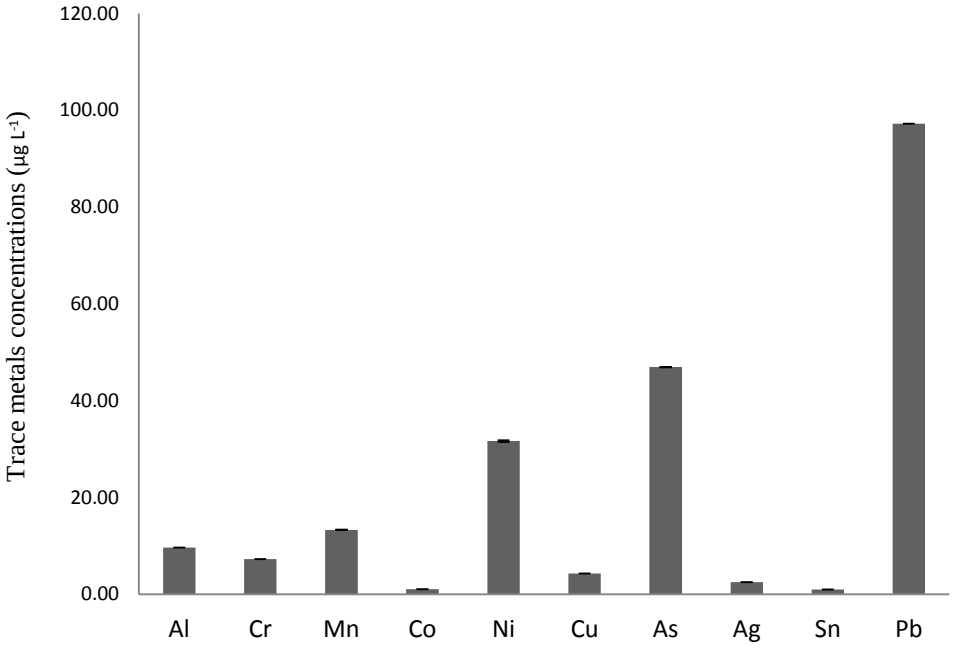

Figure 16: Heavy metals concentrations of Jeddah Red Sea near-shore sea water. The study area was performed in the central part of the eastern Red Sea coast with eight sampling areas, distributed over about 56 km stretch of Jeddah Red Sea coastal samples collected near the sea surface and results in µg L^{-1} measured by ICP-MS.

3.2.3 HEAVY METAL ANALYSIS OF SEDIMENT

According to the sediment samples collected from the off-shore water stations at about a depth of about 65 meters, Aluminium is the most abundant metal at 42.00 µg g^{-1} (ng/g dry weight of sediment) closely followed by Lead and Arsenic were the most at approximately 35.00-32.00 µg g^{-1}, respectively Figure 17. Nickle and Copper appear at 10.00-8.00 µg g^{-1} respectively, whilst other metals were found at trace levels including

Manganese at 7.00 µg g^{-1}, Chromium at 3.00 µg g^{-1}, and the majority of other metals, Cobalt, Zinc, Silver, and Cadmium at levels less than 1.00 µg g^{-1} Figure 17.

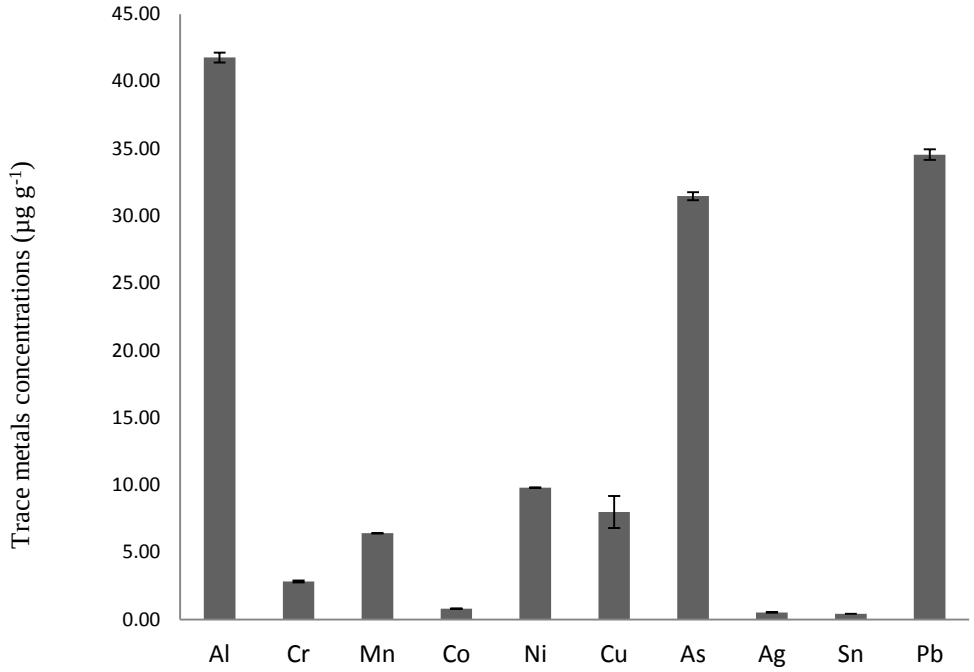

Figure 17: Heavy metals concentrations of Jeddah Red Sea sediment. Heavy metals concentrations of Jeddah Red Sea sediment. Samples at the off-shore sampling stations, acid digested measured by ICP-MS. The study area was performed in the central part of the eastern Red Sea coast with eight sampling areas, distributed over about 56 km stretch of Jeddah Red Sea coastal.

3.2.4 HEAVY METAL ANALYSIS OF PLANKTON

Plankton samples prepared from 2 L of sea water both from near- and off-shore sampling stations (See Section 2.3.3) did not yield measurements above the detection limit of the ICP-MS instrument, which is 0.001 µg L^{-1}. This finding may due to the sample collection method.

3.2.5 HEAVY METAL ANALYSIS OF FISH TISSUES

To determine whether metals ions were being tropically transferred or biomagnified into higher predators wild fish, spangled emperor *Lethrinus nebulosus* (n=5), were sampled Figure 18 from the off-shore sampling stations at around 65 m depth at night using a hook and line. Gills and liver were extracted immediately on board the fish vessel and tissues snap-frozen in liquid nitrogen for transfer back to the laboratory. Metal analysis of the tissues revealed Zinc was the most abundant at 38.91 mg g^{-1} (dry weight of tissue), followed by Aluminium at 20.00-30.00 mg g^{-1}, while the remaining metals were less 5.00 mg g^{-1}, except Nickle, which was approximately 8.00 mg g^{-1}.

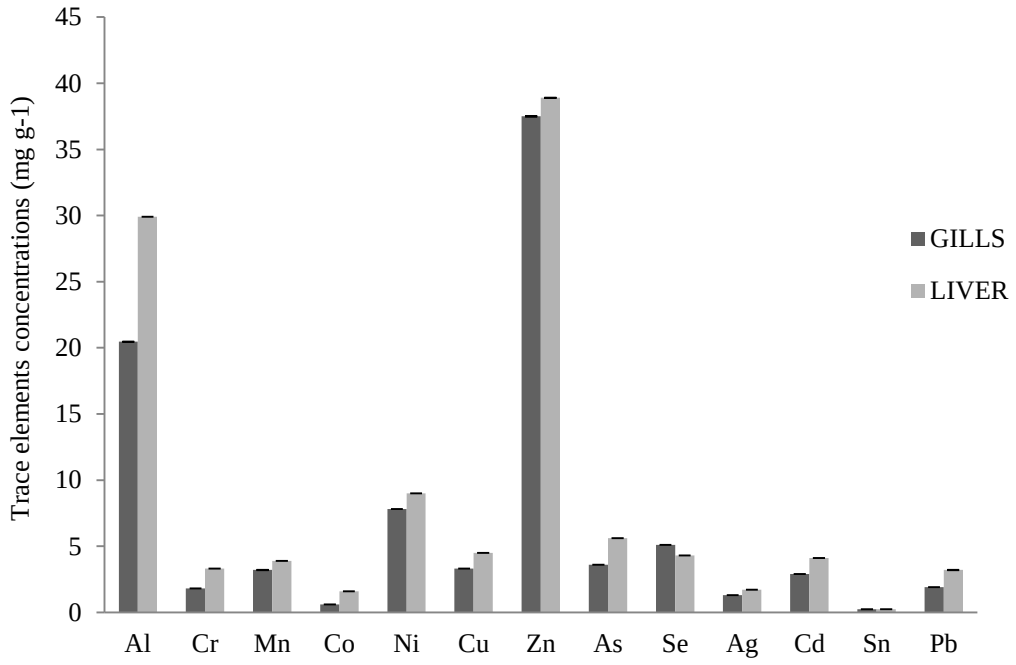

Figure 18: Heavy metals concentrations of spangled emperor fish tissues (gills & liver). Concentrations in ppb measured by ICP-MS in wet weight taken from Jeddah Red Sea in the same exact off-shore samples areas. The study area was done in the central part of the eastern Red Sea coast with eight sampling areas, distributed over about 56 km stretch of Jeddah Red Sea coastal, mg g^{-1} (dry weight of tissue).

3.2.6 HYDROCARBONS ANALYSIS AND PLANKTON

Hydrocarbons were below detectable limits in water (near- and off-shore water samples), sediments, plankton and fish samples, using the method described (See Section 2.3.5), which reflects the Gas-Chromatography instrument detection limit of 0.1 ng L^{-1}.

3.3 DISCUSSION

Natural sources, human activities and industries increased use of heavy metals has led to serious environmental pollution which specifically threatens the delicate near-shore ecosystems (Jytte Molin, 1995). The alteration in the method of sewage disposable in 2001 has resulted in a very noticeable dilution effect of the resultant pollutions impact in the shore-line surrounding Jeddah City (PERSGA, 2003), although the composition of inorganics, including Aluminium, Arsenic, Manganese, Copper, Lead and Nickle, in sediments is elevated, an effect that extends both north and south of the major sewage outflow and industrial areas of Jeddah city extending along the entire study area.

The presence of some heavy metals in sea water and sediment, create a societal health risk that, which is useful for fisheries. Fish play an important role in human nutrition and therefore need to be carefully and routinely screened to ensure that there are no high levels of heavy metals being transferred to man through their consumption. According to our current investigations of sea water samples collected from the proximity of Jeddah city from both near-shore or off-shore sampling stations that most of the metals values in the off-shore waters (Figure 14) were at higher concentrations than those found in the near-shore water samples (Figure 15), except for Aluminium, Lead and Manganese, which were higher in the near-shore waters. Also, Arsenic and Nickle in both areas appeared at similar concentrations around 44 – 32 µg L^{-1} respectively, while the Lead in the near-shore waters was at maximum value reaching 100 µg L^{-1}.

Studies over the last 20 years have confirmed the observation that heavy metals have been elevated in the Red Sea waters especially in the near-shore water samples depending on the human activities. Massoud & Fahmy (1994) and El-Ghazaly & Abdel-Aziz (2006) recorded elevated heavy metals levels of Manganese, Copper, Zinc and Cadmium, with Copper and Cadmium observed at approximately the same concentration of 1.60 and 1.10 µg L^{-1}, respectively. In our current in near-shore samples, the Copper was further elevated to around 4.30 µg L^{-1}, while Cadmium has not been detected. For Manganese and Zinc values were much higher in 1994 at 6.70 – 6.60 µg L^{-1} whilst in 2006 they had fallen to around 1.22 – 1.59 µg L^{-1}, respectively. Our results determined in the summer of 2011 show Mn to have again risen and has now exceeded its 1994 level; reaching almost 13.35 µg L^{-1}, while no Zinc has been detected. Similarly, Lead which was measured in 2006 at 7.20 µg L^{-1} was now recorded approximately 14 fold higher at 100.00 µg L^{-1}.

Many studies of heavy metals in aquatic bodies reveal that the levels of heavy metals in the sediment are usually higher than in the water, this indicates that sediments can act as sinks for heavy metals. In the collected samples from Jeddah Red Sea, the converse was observed with heavy metal values in the sea water being higher than in the sediment values; except for Aluminium concentrations, which was 17 fold greater in the sediment (42.78 µg g^{-1}) than in sea water (2.46 µg L^{-1}). Lead and Arsenic were the next highest represented metals in sediment approaching 33.00 µg g^{-1} with Nickle, Copper, Manganese and Chromium values between 2.80 µg g^{-1} and 10.00 µg g^{-1}. The remaining inorganic were represented at less than 1.00 µg g^{-1}.

In an attempt to investigate tropic transfer of metal ions into the food chain our studied investigated metal levels in both primary producers, plankton, and secondary consumers, fish. Essential metals are often transported actively across membranes

(Tamás *et al.,* 2001) and these metal transporters may also act as gateways for the entry of toxic metals into plankton (Bridges *et al.*, 2005). Frustratingly, the heavy metals within our plankton samples were below our detection limits that either reflects technical difficulties in the filtration process or the requirement for processing of larger samples of sea water. Fortunately, our collection of a local fish species, the spangled emperor *Lethrinus nebulosus*, was successful and revealed intriguing relationships with some of the metals, especially Aluminium and Zinc, recorded in associated sediments samples. *L. nebulosus* inhabits depths of between 10 and 75 metres and it is a non-migratory species is found on coral and rocky reefs, sea grass beds, mangrove swamps, as well as over sandy substrates (Drummond, 1996). In 2010, Montaser *et al.,* (2010) studied the heavy metals effects on Metallothionein expression on the fish tissues (gills and liver) in Sleek Unicorn fish (*Naso hexacanthus*) in which they identified in sea water collected samples that the maximum Copper level of around 27.00 µg L^{-1} and the Lead of 90 µg L^{-1}. However, in our collected samples of fish spangled emperor *Lethrinus nebulosus* in 2011, the Aluminium and Zinc were observed to be the highest metals in fish tissues (gill and liver). These differences may related to the collection sites and the ecology of the fish spices. The elevation of Aluminium in the fish tissue, both gills and liver tissue, is intriguing especially given the low levels observed in the sea water and the high levels recorded in the sediments. It is possible that the molluscs, echinoderms, crustaceans and polychaetes that form the majority of its diet may provide the basis for the transfer from sediment to fish but it was not possible to test this hypothesis within the context of the current study. However, when selecting metals of interest to study within mixture experiments we focused of Aluminium due to the dearth of toxicological information relating to this metal and its prevalence within the Red Sea fish tissues. We decided to combine this non-essential metal with Copper and Zinc as essential heavy metals exhibiting contrasting chemistries; Copper displaying redox potential and ability to generate oxy-radicals and

Zinc property as Lewis acid and its contribution to protein structure and cell signalling. Copper is also prominent component of the off-shore water samples, and therefore, has particular relevance to the metal dynamics occurring in this part of the Red Sea.

4 SUB-LETHAL TOXICITY ASSESSMENT OF COPPER IN *CAENORHABDITIS ELEGANS*

4.1 INTRODUCTION

Copper (Cu) is an essential micro-nutrient. The metabolism and health impacts of Copper have been previously comprehensively reviewed (Hughes and Stürzenbaum, 2007; Yoon *et al.,* 2008; Kakkar and Jaffery, 2005). In humans Copper is necessary for the development of connective tissue, nerve coverings, and bone (Fraga, 2005). Copper also participates in both Iron (Fe) and energy metabolism. Copper acts as a reductant in the enzymes such as superoxide dismutase, cytochrome oxidase, lysil oxidase, dopamine hydroxylase, and several other oxidases that reduce molecular oxygen (Speisky *et al.,* 2009). In humans, gastrointestinal absorption of Copper is normally regulated through homeostatic mechanisms and it is transported through serum originally bound to albumin and later more firmly to ceruloplasmin and transcuprein (Yoon *et al.,* 2008).

These elegant homeostatic pathways are designed to supply Copper to those system where it is required but to prevent it elevating beyond acceptable thresholds, because once outside these levels, Copper exhibits significant toxicology. The pathology of Copper toxicology presents as nausea, vomiting and diarrhoea, and ultimately, Copper exposure may result in necrosis and death (Fraga, 2005). Extreme accumulation of Copper in liver, brain, kidneys and cornea occurs in the genetic disorder Wilson's disease, where the activity of the liver vascular Cu-ATPase is disrupted (Sugimoto *et al.,* 1988). Also the biochemical and morphological observations made in the Long-Evans Cinnamon (LEC) rats suggest that it is an authentic model of Wilson's disease (Li *et al.,* 1991; Wu *et al.,* 1994).

The cellular mechanism underlying Copper toxicology is associated with its activity as a superoxide generator, and thus, the ability to induce oxidative stress. It can induce the formation of hydrogen peroxide (H_2O_2), which has the potential to be transformed to hydroxyl radical OH (Eisler, 1998); a molecule that is extremely reactive and modifies all biomolecules in its vicinity, including lipids, proteins and DNA (Prato *et al.*, 2013).

The most sensitive effect of Copper overload in vertebrates appears to be cholesterol synthesis (Huster *et al.*, 2007). Copper deficiency in humans is rare, but when it occurs leads to normocytic, hypochromic anemia, leucopenia and neuropenia, and inclusive osteoporosis in children (Kanumakala *et al.*, 2002). Excessive dietary Zinc can cause Copper deficiency. Copper is involved in the regulation of antioxidant activities, such as the activity of (Cu, Zn-SOD), and low dietary Copper intake reduces glutathione peroxidase activity. It has been believed for a long time that oxidative stress is responsible for Copper deficiency induced cardiac hypertrophy and dysfunction. Copper is an important regulator of mitochondrial function and is required for cytochrome C oxidase (CCO) activity, rendering it essential for oxidative phosphorylation.

Copper levels are observed as the most abundant metal ion in the sea water from the off-shore sampling stations. It is important to consider the toxicity of Copper individually in order to interpret the possible combinatorial toxicology of mixtures containing Copper. After establishing the impact of Copper on life history traits (mortality and reproduction), we assessed the molecular mechanisms underlying Copper toxicity by evaluating changes in the composition of metabolites and holistic metal levels during expose to sub-lethal doses of Copper in our test sentinel species, the nematode *C. elegans*.

4.2 RESULTS

4.2.1 MORTALITY

To derive the lethality profile of Copper within out test system we performed 24 hours exposures of adult nematodes (See Section 2.4.8) to concentrations of Copper estimated from the literature as ranging between the $LC_{10} - LC_{40}$ (Table 5; Chu *et al.*, 2002). Comparing the effect of different doses of Copper on the mortality after 24 hours exposures on *C. elegans* (one-way ANOVA) revealed no significant effect of Copper on the mortality of *C. elegans* at the ($p < 0.05$) Figure 19. However, the sub-lethal Copper doses used demonstrated a slight impact on the mortality Figure 19 with ~10% of *C. elegans* population not surviving the 24 hours exposure in comparison to a 100% survival of control organisms. The discrepancy between expected and observed survival rates is explained by differences in the time and temperature used for these observation, which was 24 hours and 20^{o}C rather than the 48 hrs and 22^{o}C used when the original LC_{50} was determined (Section 2.4.8). A temporal comparison of mortality is described in Chapter 7.2.1.

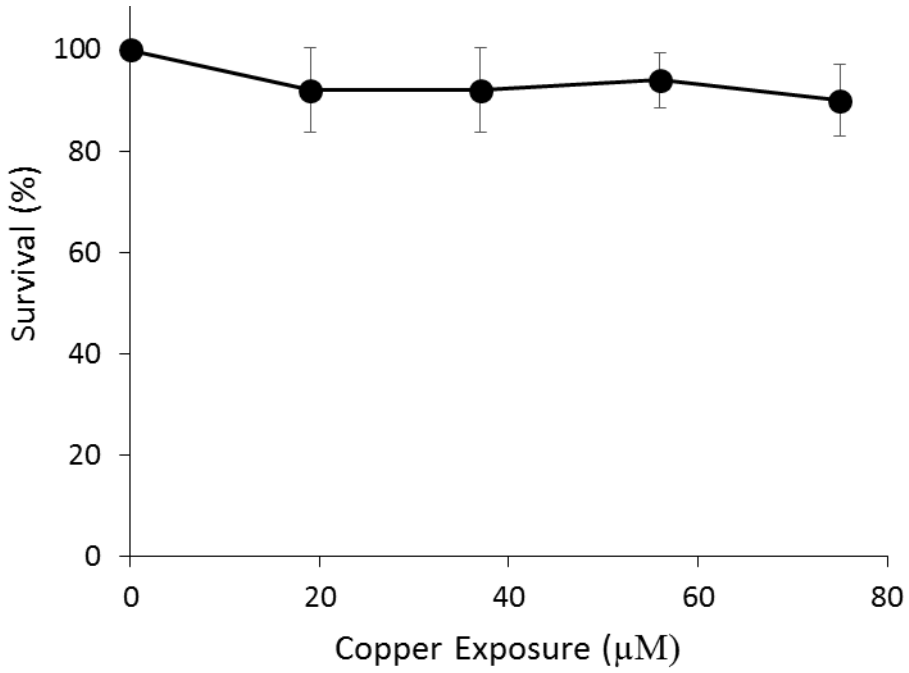

Figure 19: The mortality of *C. elegans* in numbers after Copper exposure for 24 hours. 10 adults nematodes from age-synchronized cultures exposed to four different concentrations and used for testing were placed in 1 ml of test solution. The number of survivals was counted. For each test concentration and control, the average number of survivals from the wells was obtained for each trial, and at least five trials were conducted after their exposure to different doses of Copper in a 24 hours period. Points represent mean ± SD.

4.2.2 REPRODUCTION

To evaluate the sub-lethal impact of Copper on reproduction, we performed a 72 hours exposure using age synchronized cultures (See Section 2.4.9) and employing the identical exposures levels based on previous literature and described in Section 2.4.6 above. A comparison of the effect of Copper on the number of eggs laid by *C. elegans* (one-way ANOVA) revealed a significant effect ($p < 0.05$) for all of the four different concentrations used Figure 20. A further *post hoc* comparison (using the Tukey's HSD test) indicated that these were significantly different than the control condition Figure 20. *C. elegans* displayed ~70% reduction in egg production at all levels of Copper used and

although there was a trend for egg number to reduce further with exposure to the higher Copper levels this reduction was not statistically significant when compared to the minimum dose used.

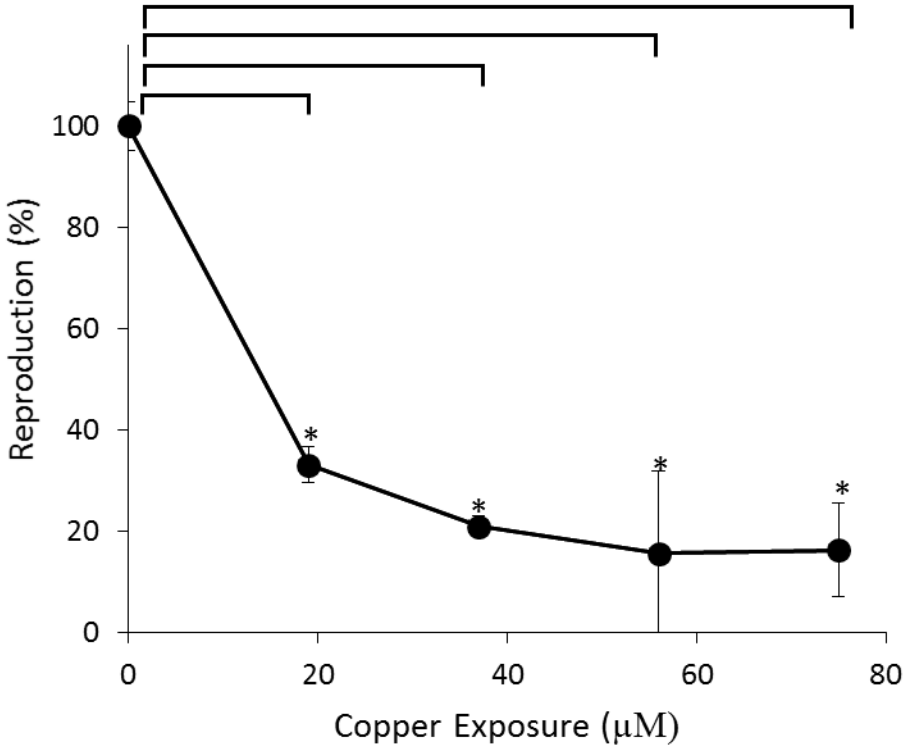

Figure 20: Number of *C. elegans* eggs laid after Copper exposure for 72 hours. Single nematode from age-synchronized cultures exposed to four different concentrations and used for testing were placed in 1 ml of test solution. The number of offspring at all stages beyond the eggs was counted. For each test concentration and control, the average number of progenies from the wells was obtained for each trial, and at least five trials were conducted. Points represent mean ± SD. * represents significant compared to the control via t-test ($p < 0.05$).

4.2.3 METABOLOMICS

4.2.3.1 AMINO ACIDS

The potential impact of Copper on metabolism may result from either the metabolic demands of detoxification or as a result of the adverse impact of excess Copper. The intracellular chaperones involved in binding Copper within the cytosol of *C. elegans* include the metabolite Glutathione, the phytochelatin peptides and the protein metallothionein. The Cystathionine pathway (See Section 1.6, Figure 5) is key to the production of these chaperones through the generation of Cysteine and Methionine and therefore, it is important to evaluate the intermediates within this pathway (Rao *et al.*, 2005). It is therefore intriguing that in the two highest sub-lethal Copper doses we observed elevated Cystathionine and Cysteine levels Table 6 and Figure 21.

The production of Reactive Oxygen Species (ROS) is the central mechanism by which Copper impacts on the cell and it may be assumed that this process will directly impact on the synthesis of an array of amino acids. It has been noticed that in most of the Copper concentrations Glycine and Tryptophan have been reduced, while β-aminoisobutyric acid (BAIBA) and Histidine were increased.

Table 6: A list of differentially regulated aqueous phase metabolites observed in adult nematodes after Copper exposure. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test was compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample was for the significant values.

Cu 19 µM		Cu 37 µM		Cu 56 µM		Cu 75 µM	
Glycine	↓	Glycine	↓	Glycine	↓	Glycine	↓
Valine	↓	BAIBA	↑	Valine	↑	AABA	↑
BAIBA	↑	Threonine	↓	BAIBA	↑	Hydroxyproline	↑
Leucine	↓	Glutamine	↓	Leucine	↑	Phenylalanine	↑
Allo Isoleucine	↓	Glycine	↓	Allo Isoleucine	↑	Histidine	↑
Isoleucine	↓	Histidine	↑	Isoleucine	↑	Tyrosine	↑
Tryptophan	↓	Tryptophan	↓	Threonine	↓	Proline	↑
				Methionine	↑	Cystathionine	↑
				Hydroxyproline	↑	Cysteine	↑
				Phenylalanine	↑		
				Glutamine	↓		
				Histidine	↑		
				Tyrosine	↑		
				Proline	↑		
				Tryptophan	↓		
				Cystathionine	↑		

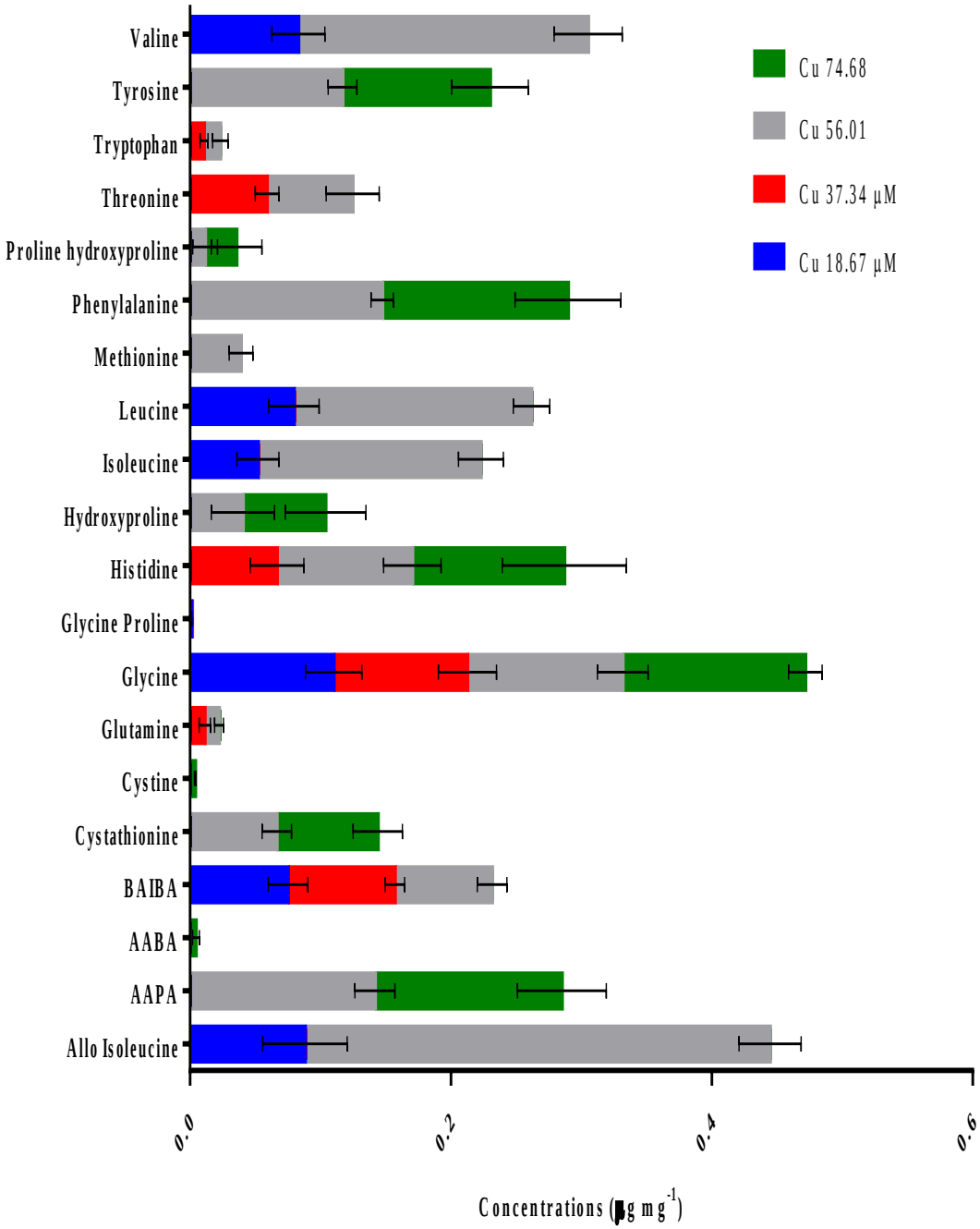

Figure 21: Changes in aqueous phase (Amino Acids) concentrations measured by ICP-MS. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test has made and compared to the control via t-test ($p < 0.05$) for significance after different Copper dose exposures for 24 hours; compared to control samples. All bars are mean ± SD in the chart and each bar represents significant difference when compared to control levels ($p < 0.05$).

4.2.3.2 FATTY ACIDS

Previous studies have revealed that chronic Copper exposures affect some organisms by decreasing its total fatty acids content. On the other hand, some organisms may retain essential fatty acids of n3 and n6 series (Maazouzi *et al.*, 2008). Generally our findings show similar affects showing a marked decrease in the total fatty acids content in *C. elegans* with the exception of C18:2n6, which increases with exposure Table 7 and Figure 22.

Table 7: A list of differentially regulated organic phase metabolites observed in adult nematodes after Copper exposure. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Cu 19 µM		Cu 37 µM		Cu 56 µM		Cu 75 µM	
C13:0	↓	C13:0	↓	C14:0	↓	C14:0	↓
C14:0	↓	C14:0	↓	C15:0	↓	C15:0	↓
C17:0	↓	C15:0	↓	C18:2n6	↑	C17:0	↓
C18:0	↓	C17:0	↓	C20:4n6	↓	C18:0	↓
C18:1n9	↓	C18:1n9	↓	C21:0	↓	C18:2n6	↑
C18:2n6	↑	C18:2n6	↑	C22:0	↓	C20:0	↓
C20:4n6	↓	C20:4n6	↓			C20:4n6	↓
C20:5n3	↓	C21:0	↓			C21:0	↓
		C20:5n3	↓				
		C22:0	↓				

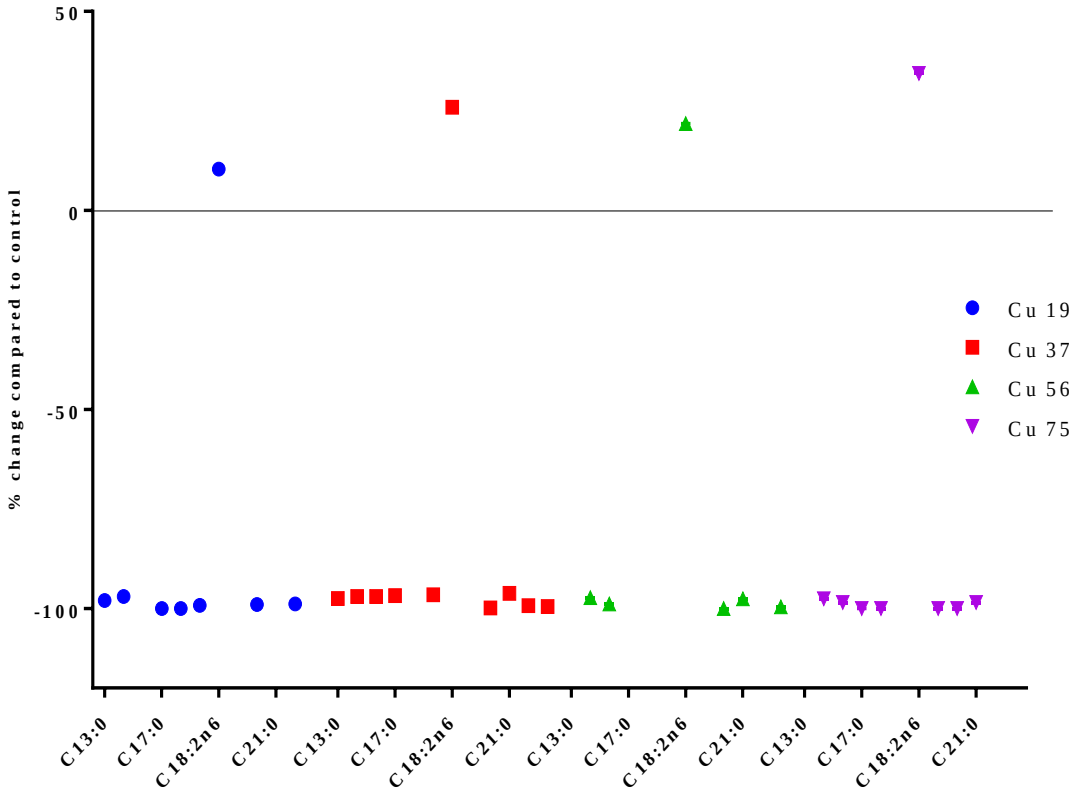

Figure 22: Changes in Fatty Acids composition measured by GC-MS. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 h period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance after different Copper dose exposures for 24 hours. All points are mean percentage change from control ± SD in the chart and each point represents significant difference when compared to control levels ($p < 0.05$).

4.2.3.3 IONOMICS

The Ionomic analysis provides a comprehensive approach and allows simultaneous measurement of most of the relevant components under study that are present within the same class of molecules. A number of studies have been carried out for observing the effects of different elements on the human body and reported that Copper supplementation can aid in decreasing the toxic effects of some heavy metals, as well as inhibiting their absorption (Watts, 1989).

Under the Copper exposures, time and dose, employed within this study we see minimal mortality suggesting that not acutely toxic for Copper and this is reinforced when we consider the relationship between exposure and body burden, which showed a linear relationship with a gradient of ~1 Figure 23C. This indicated that the flux of Copper was not being actively moderated, neither by reducing influx through regulating Copper transporters, such as the Ctr (SLC31) family, or by increasing efflux. The impact of the increased Copper and moderation of Copper flux is reflected in changes in body burden of a range of metals Table 8 & Figure 23A including the essential micronutrient Magnesium and Manganese, which showed ~2 fold reductions at the highest Copper concentration Figure 23D. Although some other metals such as Aluminium and Arsenic showed very modest increases of ~1.2-1.5 Figure 23B fold, these were modest probably physiologically not significant. Detailed information regarding the elemental composition changes in *C. elegans* after different sub-lethal Copper doses compared with the control sample (after statistics steps) has been taken to reveal significant metals that have been affected and changed (See appendix Table 17).

Table 8: A list of identified elemental composition measured by ICP−MS of adults nematodes Copper exposed. Nematodes were from age-synchronized cultures exposed to four different concentrations and for each test concentration and control, the average from each test was obtained for each trial, and at least five trials were conducted after their exposed with different doses of Copper in 24 h period. ICP−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values. Data after the ANOVA test was compared to the control via t-test ($p < 0.05$) for significance.

Cu 19 µM		**Cu 37 µM**		**Cu 56 µM**		**Cu 75 µM**	
Ag	↓	Ag	↓	Cd	↓	Cd	↓
Cd	↓	Be	↓	Cs	↓	Cs	↓
Cs	↓	Bi	↑	Mg	↓	Lu	↑
Li	↑	Co	↑	Mn	↓	Mg	↓
Mg	↓	Cs	↓	Se	↓	Mn	↓
Mn	↓	Dy	↑			P	↓
Se	↓	Er	↑			Se	↓
		Eu	↑			Sn	↑
		Ga	↓				
		Gd	↑				
		Ho	↑				
		La	↑				
		Lu	↑				
		Nb	↑				
		Nd	↑				
		Pr	↓				
		Rb	↓				
		Sb	↓				
		Se	↓				
		Sm	↑				
		Tb	↑				
		Th	↑				
		Tl	↑				
		Tm	↑				
		U	↓				
		V	↓				
		W	↓				
		Y	↑				
		Yb	↑				

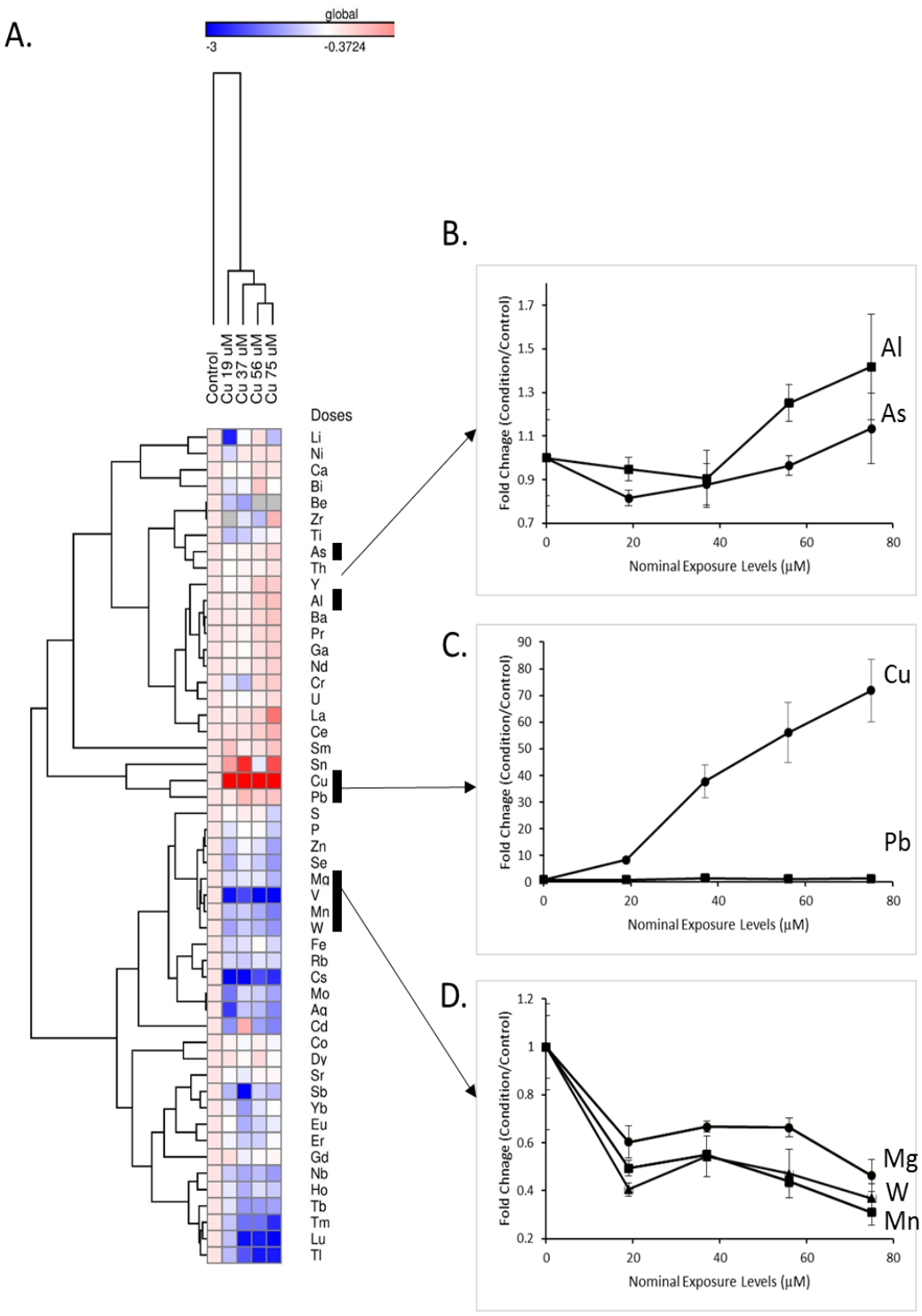

Figure 23: Changes in elemental composition measured by ICP-MS of Copper exposed nematodes. Nematodes were from age-synchronized cultures exposed to four different concentrations of Copper and for each test concentration and control, the average from each test was obtained for each trial, and at least five trials were conducted after their exposed with different doses of Copper in 24 hours period. **Panel A:** Hierarchical clustering of changes in ions levels in response to Copper exposure. Log_2 (Mean fold change) is clustered for ion and condition using a minus one Pearson correlation with average linage method applied. **Panels B-C:** Example individual metal ion fold changes in response to Copper exposure. All points are mean with error bars indicating ± SE.

4.2.4 SYSTEMS LEVEL ANALYSIS OF COPPER IMPACT

In order to derive the relationships between the impact of Copper at different levels of biological organisation to determine metabolite or ion of most significance on the life history traits, we constructed linear models to predict continuous linear relationships between the Copper exposure (target) and one or more molecular/life history parameters (predictors).

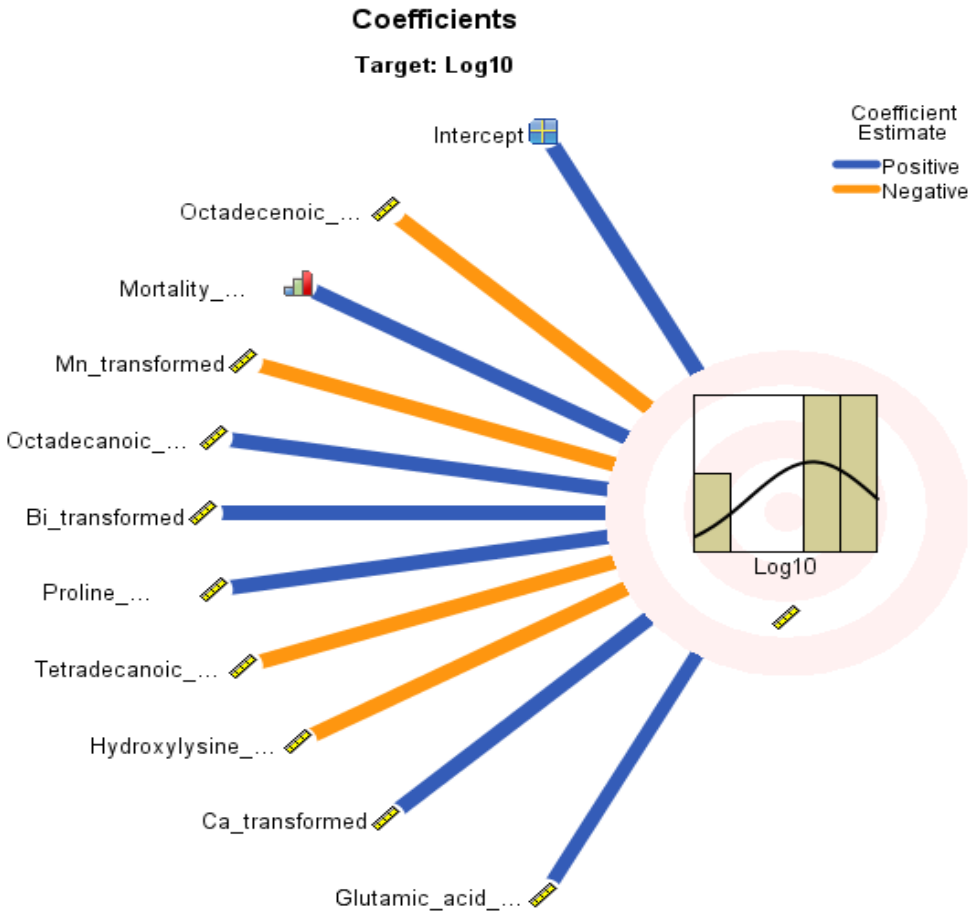

Figure 24: Coefficients (SPSS Linear Models) for Copper exposure and most important effects. This chart displays the intercept first, and then sorts effects from top to bottom by decreasing predictor importance. Within effects containing factors, coefficients are sorted by ascending order of data values. Connecting lines in the diagram are coloured based on the sign of the coefficient (Positive or Negative) and weighted based on coefficient significance, with greater line width corresponding to more significant coefficients (smaller *p*-values). The target is Copper exposure concentrations (Log 10).

According to the automatic linear modeling, we may categorize the top 10 important predictors to main classes (Positive and Negative) that related to the different Copper exposures depending on the estimating coefficient as follow:

- Positive Predictors: Mortality, C18:0, Bismuth, Proline, Calcium and Glutamic acid.
- Negative Predictors: C18:1n9, Mn, C14:0, and Hydroxylysine.

Comparing results from the new SPSS feature (Automatic Linear Modeling) with the most acceptable statistic tests (ANOVA and t-test), we can reject some of these predictors that do not match such as: Mortality and C18:0, as these parameters were decreasing with Copper exposures.

4.3 DISCUSSION

Our mortality and reproduction data revealed that this impairment could be due to a reduction in the amount of metabolic energy available in the animal, as previous work has shown Copper to impact on mitochondrial function therefore reducing cellular energy production (Bundy *et al.,* 2008). Copper is a superoxide generator, and can induce oxidative stress in tissues. It has ability to induce the formation of hydrogen peroxide (H_2O_2), which might be transformed to hydroxyl radical OH, which is extremely reactive and modifies all biomolecules in its vicinity, including lipids, proteins and DNA (Prato *et al.,* 2013).

Our measurements of the changes in amino acid composition in response to Copper exposure revealed that proline had increased at the highest Copper dose. Kebeish *et al.,* (2014) observed copper dependent increase in the intracellular Proline pool in *C. vulgaris* and suggested that this could be considered as an adaptive mechanism since it causes a reduction in the level of accumulated NADH and H^+; an observation that had previously been reported by Rodríguez-Montelongo *et al.,* (2006). There are two sites of electron transfer to membrane bound Copper, coincident with two major loci of Copper mediated damage by H_2O_2 that have been identified in the respiratory chain. One site of Copper reduction is localized between NADH and Quinone and the other between Quinone and the cytochromes. Therefore by reducing the accumulation of NADH, Proline suppresses Copper mediated damage of the electron transport chain. It is also proposed that elevated Proline acts as a carbon and nitrogen source facilitating rapid stress recovery. It also serves as a plasma membrane and macro-molecule stabilizer which together act as a free radical scavenger, thereby mitigating the impact of Copper toxicity (Kebeish *et al.,* 2014; Rodríguez-Montelongo *et al.,* 2006).

Other findings revealed by our amino acids analysis was the elevation of Cystathionine at the two highest Copper exposure levels. In addition, it has been noticed that cysteine and methionine both increase in response to Copper. Cystathionine is the key intermediate in the trans-sulfuration pathway, a reaction by which Cysteine and Methionine can be generated (Rao *et al.,* 2005). It is an important pathway as it is the only process by which cysteine, a non-essential amino acid is generated and requires the use of the essential amino acid methionine (Houghton & Cherian, 1991). The Trans-sulfuration pathway Figure 5 involves the two key enzymes Cystathionine Y-lyase (CγL) and Cystathionine-β-synthase (CβS) which together keep a balance in the production of Cystathionine and Cysteine. Previous studies in the insect *Chironomus tepperi* has shown Copper to induce CγL whilst suppressing CβS (Jeppe *et al.,* 2014), thereby biasing the pathway to generate Cysteine. However, previous work in *C. elegans* support our findings, demonstrating the elevation of both Cystathionine and Cysteine, since they are key to the generation of phytochelatin and metallothionein respectively; the two key Copper detoxification pathways (Hughes *et al.,* 2009). The difference observed between insect response and that of nematodes reflects the finding that arthropods do not have phytochelatin synthase whilst nematodes do (Bundy & Kille, 2014).

For fatty acids analysis, we have noticed generally that Copper has supressed the production of most of the fatty acids. According to Maazouzi *et al.,* (2008) chronic Copper exposure affects *D. villosus* by decreasing its total fatty acids content. In contrast, this crustacea retained the levels of the essential fatty acids of n3 and n6 series. This finding may be a key factor in its invasive success of contaminated systems (Rikans & Hornbrook, 1997) since lipid peroxidation is a chain reaction between polyunsaturated fatty acids (PUFA) and ROS, and it produces both lipid peroxides and hydrocarbon polymers that are highly toxic to the cell. It is considered to be a major phenomenon by which oxyradicals can cause tissue damage leading to impaired cellular function and

alterations in the physicochemical properties of cell membranes, which in turn disrupt vital functions (Maazouzi *et al.*, 2008; Rikans & Hornbrook, 1997).

5 SUB-LETHAL TOXICITY ASSESSMENT OF ZINC IN *CAENORHABDITIS ELEGANS*

5.1 INTRODUCTION

The essential role of Zinc is demonstrated by the deleterious effects of Zinc deficiency and by the link between Zinc regulatory dysfunction and the pathophysiology of various disease states, including neurodegeneration, inflammation, diabetes, cancer. Recent data reinforces zinc's essential role, confirming its widespread involvement in development, immunity, reproduction, endocrinology, and neurotransmission (Hogstrand *et al.,* 2009).

Hogstrand *et al.,* (1996) in his study on freshwater rainbow trout has stated that Zn^{2+} was found to be a potent blocker of basolateral transporter for Zinc, causing a mixed inhibitory effect on the ATP-driven Ca^{2+} transport at a free Zinc activity. (Glover *et al.,* (2004) reported that equimolar calcium nitrate significantly retarded the passage of Zn^{2+} across the intestinal epithelium, and therefore, it is possible that calcium and zinc share uptake pathways in piscine intestine, similar to that of the gill.

Zinc acts as a cofactor for an estimated 3,000 human proteins, representing 10% of the genome, and has a well-established role in regulation of gene expression through metal-responsive transcription-factor-1 (MTF1) (Hogstrand *et al.,* 2008). There are 2–3 g of Zinc present in the human body and about 1 mg/L in plasma. Zinc deficiency is common in underdeveloped countries and is mainly associated with malnutrition, affecting the immune system, wound healing, the senses of taste and smell, and impairing DNA synthesis. Zinc seems to support normal growth and development in pregnancy, childhood, and adolescence. Zinc toxicity has been seen in both acute and chronic forms.

Intakes of 150–450 mg of Zinc per day have been associated with low Copper status, altered Iron function, reduced immune function, and reduced levels of HDL (Osredkar, 2011).

Zinc is an important constituent of the body as it aids in a number of activities and processes in the body; but in excess, it can be harmful and *C. elegans* have been proved to be a useful model to study these effects. It has previous been observed that if these nematodes are exposed to 0.2 mM of Zinc, the development of the nematodes is arrested. It was also shown that in the presence of Zinc 0.2 mM reproduction was considerably 'slowed', egg laying frequency reduced, and at higher concentrations of Zinc (0.3 mM) the reproduction was fully inhibited (Bruinsma *et al.,* 2008; Chu *et al.,* 2002). It was important to confirm these morphological observations within our experimental systems and therefore, enable us to integrate these finding with impacts to the metabolome and global ion levels.

5.2 RESULTS

5.2.1 MORTALITY

To derive the lethality profile of Zinc within our test system we performed 24 hours exposure of adult nematodes (See Section 2.4.8) to concentrations of Zinc estimated from the literature as ranging between the $LC_{10} - LC_{40}$ (Table 5; Chu *et al.*, 2002). Comparing the effect of different doses of Zinc on the mortality of *C. elegans* a one-way ANOVA between subjects was conducted. This revealed a significant effect of the amount of Zinc on mortality of *C. elegans* at the ($p < 0.05$) level when exposed to Zinc at 1019 µM and 1358 µM in comparison to controls Figure 25. This observation was confirmed using *post hoc* comparisons using the Tukey's HSD test and independent samples t-test both showed that survival was significantly different when exposed to the two high doses of Zinc. Specifically, our results suggest that when *C. elegans* was exposed to >1 mM Zinc for 24 hours the mortality decreased. Although, the percentage of nematode mortality did not exceed 15% when compared with control sample. The discrepancy between expected and observed survival rates could be explained by differences in the time and temperature used for these observation, which was 24 hours and 20^{o}C rather than the 48 hours and 22^{o}C used when the original LC_{50} was determined (Section 2.4.8). A temporal comparison of mortality has been described in Chapter 7.2.1.

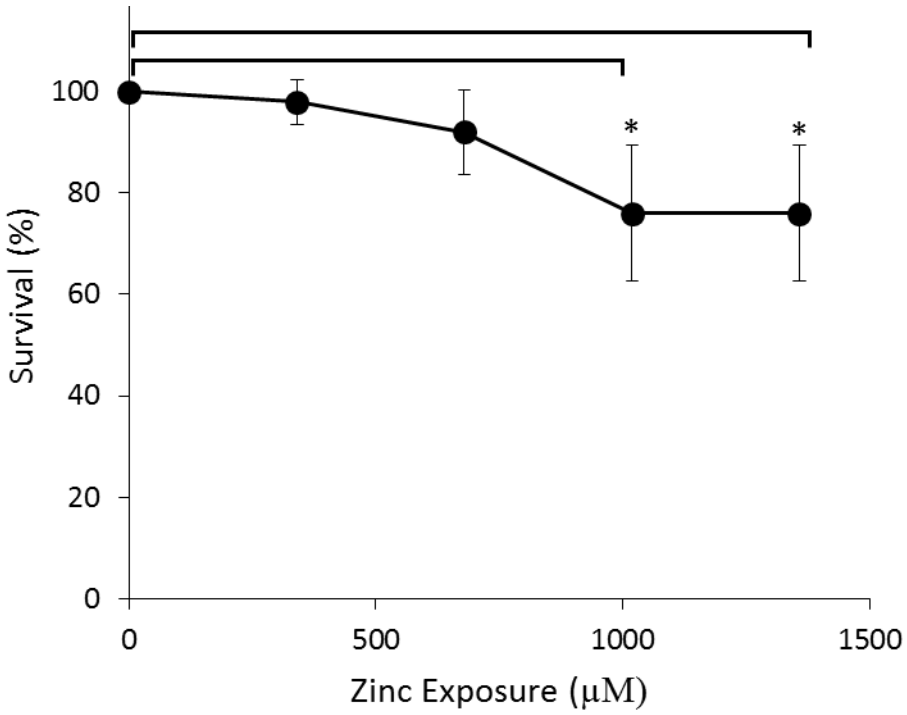

Figure 25: The survival statistics of *C. elegans* after Zinc exposure for 24 hours. 10 adults nematodes from age-synchronized cultures exposed to four different concentrations and used for testing were placed in 1 ml of test solution. The number of survivals was counted. For each test concentration and control, the average number of survivals from the wells was obtained for each trial, and at least five trials were conducted after their exposed with different doses of Zinc in 24 hours period. T-test comparisons are indicated by square brackets above the relevant bars and * shows a p <0.05. Points represent mean ± SD.

5.2.2 REPRODUCTION

To evaluate the sub-lethal impact of Zinc on reproduction we performed a 72 hours exposure using age synchronized cultures (See Section 2.4.9) and employing the identical exposures levels based on previous literature and described above in Section 4.2.1. A comparison of the effect of Copper on the number of eggs laid by *C. elegans* (one-way ANOVA) revealed that there was a significant effect of amount of Zinc on egg laid number of *C. elegans* at the $p < 0.05$ level for all four of the exposure concentrations. In addition, *post hoc* comparisons using the Tukey's HSD test indicated they were significantly different from the control condition. These results confirm that reproduction is relatively sensitive to Zinc exposure in *C. elegans* with exposure to levels >300 µM causing a significant decrease in fertility.

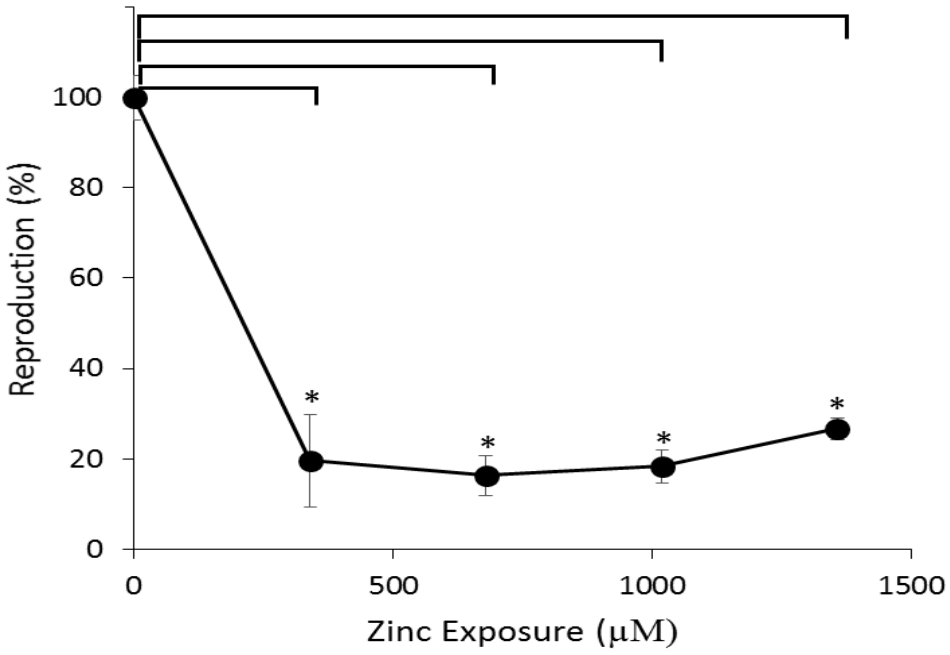

Figure 26: Number of *C. elegans* eggs laid after Zinc exposure for 72 hours. Single nematode from age-synchronized cultures exposed to four different concentrations and used for testing were placed in 1 ml of test solution. The number of offspring at all stages beyond the eggs was counted. For each test concentration and control, the average number of progenies from the wells was obtained for each trial, and at least five trials were conducted. Error Bars represent mean ± SD. T-test comparisons are indicated by square brackets above the relevant bars and * represents significant compared to the control via t-test ($p < 0.05$).

5.2.3 METABOLOMICS

5.2.3.1 AMINO ACIDS

The human body contains 2–3 g Zinc, and nearly 90% is found in muscle and bone (Wastney *et al.,* 1986). Other organs containing estimable concentrations of Zinc include prostate, liver, the gastrointestinal tract, kidney, skin, lung, brain, heart, and pancreas (Llobet *et al.,* 1988). Oral uptake of Zinc leads to absorption throughout the small intestine and distribution subsequently occurs via the serum, where it predominately exists bound to proteins (Maret, 2013). On the cellular level, 30–40% of Zinc is localized in the nucleus, 50% in the cytosol and the remaining part is associated with membranes. Cellular Zinc underlies an efficient homeostatic control that avoids accumulation of Zinc in excess (Plum *et al.,* 2010). Several dietary factors can influence Zinc absorption, including other trace elements (e.g., Cu, Fe, Pb, Ca, Ca, Co) amino acids, simple and complex carbohydrates, and protein. For humans, Phytate in diets is the main determinant of Zinc absorption. In general, low molecular weight substances, such as amino acids, increase the absorption of Zinc (Wapnir & Stiel, 1988). Imidazole, Tryptophan, Proline, and Cysteine have also been shown to increase Zinc absorption from various regions of the gastrointestinal tract. Wapnir & Stiel (1988) suggested that this increase was due to the presence of both mediated and non-mediated transport mechanisms for amino acids.

In our experiments, sub-lethal exposure of *C. elegans* to Zinc caused a general increase in most metabolites (amino acids) except for BAIBA, Leucine, Isoleucine and Glutamic acid which decrease Table 9 and Figure 27.

Table 9: A list of differentially regulated aqueous phase metabolites observed in adult nematodes after Zinc exposure. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Zn 340 µM	Zn 679 µM	Zn 1019 µM	Zn 1358 µM
BAIBA ↓	Glycine ↑	Cystathionine ↑	Threonine ↑
	Glycine Proline ↑	Allo Isoleucine ↓	Tyrosine ↑
	Phenylalanine ↑	AAPA ↑	Allo_Isoleucine ↑
	Proline ↑	Glycine ↓	BAIBA ↓
	Threonine ↑	Hydroxyproline ↑	Cysteine ↑
	Tyrosine ↑	Isoleucine ↓	Glutamic acid ↓
	Valine ↑	Leucine ↓	Glutamine ↑
	Allo Isoleucine ↑		Histidine ↑
	Cysteine ↑		Hydroxyproline ↑
	Histidine ↑		Isoleucine ↑
	Isoleucine ↑		Leucine ↑
	Leucine ↑		Lysine ↑
	Methionine ↑		Methionine ↑
			Phenylalanine ↑
			Serine ↑

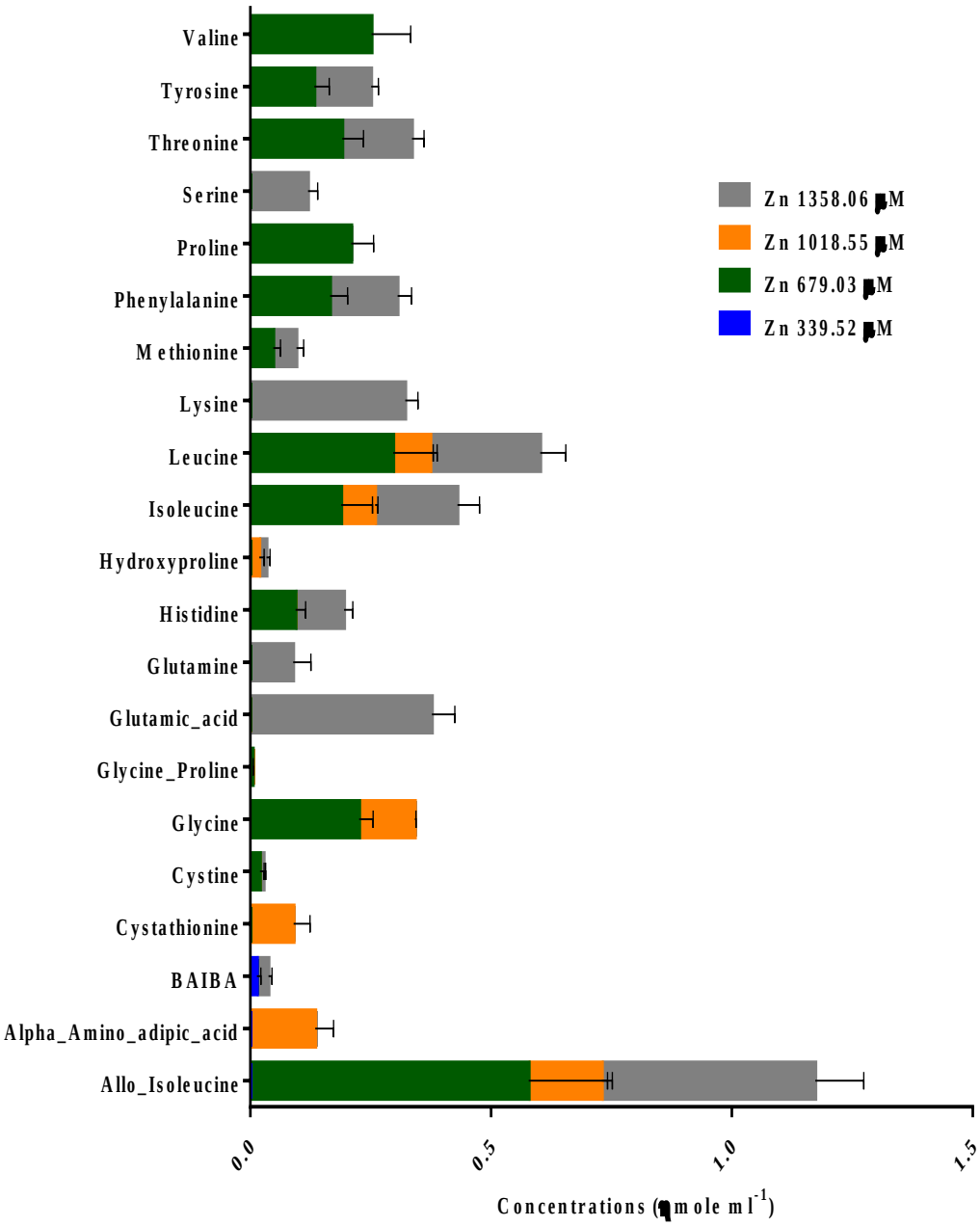

Figure 27: Changes in aqueous phase (Amino Acids) concentrations of Zinc in adult nematodes measured by ICP-MS. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance after different Zinc dose exposures for 24 hours. All bars are mean ± SD in the chart and each point represents significant difference when compared to control levels ($p < 0.05$).

5.2.3.2 FATTY ACIDS

Zinc induces the synthesis of metallothioneins which is a factor in regulating the metabolism of Zinc including absorption and storage (Miles *et al.,* 2000). Zinc is a functional component of estimating 3,000 proteins that contribute to gene expression and regulation of genetic activity. The deficiency symptoms of Zinc and essential fatty acids, which include growth retardation, delayed sexual maturation, infertility, dermal lesions, alopecia and decreased rate of wound healing, show remarkable similarities. This evoked the possibility of a mutual interaction between Zinc and essential fatty acids. This hypothesis is sustained by decreased Δ-5 and Δ-6 desaturase activity in various tissues of Zn-deficient rats (Cunnane *et al.,* 1984); an observation that has not been verified by others (Kramer *et al.,* 1996; Eder & Kirchgessner, 1996).

Our findings, presented in Table 10 and Figure 28, revealed that Zinc had a remarkable effect on the nematode fatty acids profile, decreasing the amount of all fatty acids except the C18:0, which increased in amount when compared with control samples.

Table 10: A list of differentially regulated organic phase metabolites observed in adult nematodes after Zinc exposure. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Zn 340 µM		Zn 679 µM		Zn 1019 µM		Zn 1358 µM	
C14:0	↓	C14:0	↓	C20:0	↓	C20:0	↓
C20:0	↓	C18:2n6	↓	C20:4n6	↓	C20:4n6	↓
C20:4n6	↓	C18:0	↑	C21:0	↓	C21:0	↓
C21:0	↓	C20:0	↓	C17:0	↓	C17:0	↓
C17:0	↓	C20:4n6	↓	C18:2n6	↓	C18:2n6	↓
C18:2n6	↓	C17:0	↓	C18:0	↑	C18:0	↑
C18:0	↑						

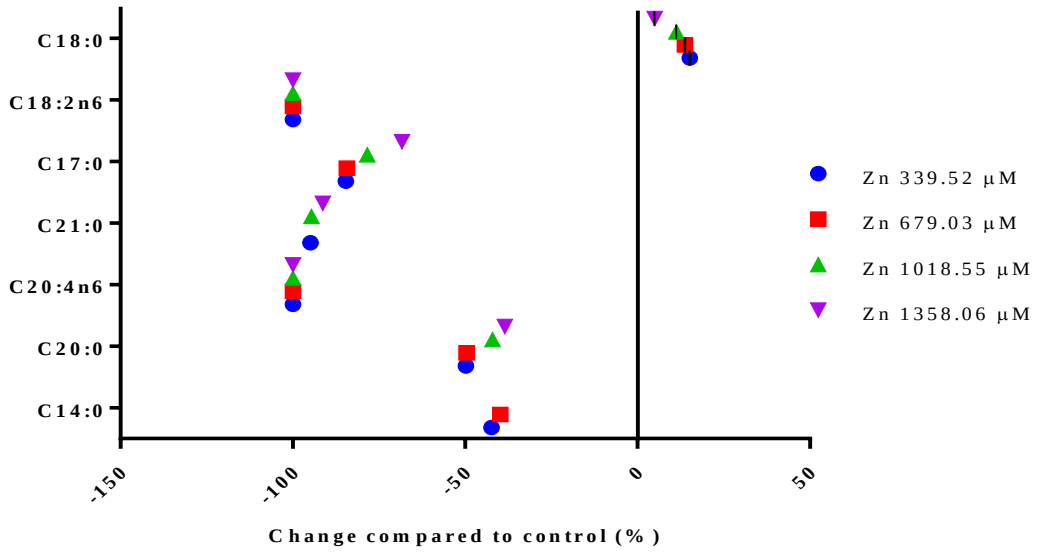

Figure 28: Changes in Fatty Acids composition measured by GC-MS of Zinc exposed nematodes. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance after different Zinc dose exposures for 24 hours. All points are mean percentage change from control ± SD in the chart and each point represent significant difference when compared to control levels ($p < 0.05$).

5.2.3.3 IONOMICS

Our results confirmed that Zinc exposure has a significant impact on internal elemental balance in *C. elegans* Table 11 and Figure 29. It is intriguing that over the concentration range employed, the body burden resulting from Zinc exposure increase of in a linear fashion Figure 29A. Given the elegant homeostatic influx and efflux mechanisms provide by the Zinc transporters Zip (SLC39) and Znt (SLC30), respectively Cousins *et al.,* (2006), we may have predicted that at sub-lethal exposures the internal Zinc levels would have been maintained relatively constant. However, there was a gradual increase of body burden with increasing exposure, indicated by 2:1 relationship between body burden and exposure Figure 29A. The secondary impacts of Zinc exposure included increased amounts of Silver and Phosphorus, the majority of the remaining ions level were seen to decrease. Detailed information regarding to the elemental composition change in *C. elegans* after different sub-lethal Zinc doses compared with the control samples and together with detail statistical analysis can been found in the appendix Table 18.

Zinc is a nutrient that is essential for all life. Zinc has roles in many biological processes; protein-bound Zinc contributes to enzymatic activity and protein structure, and labile Zinc functions in signal transduction (Roh *et al.,* 2012). Excess Zinc is also deleterious, since it may displace other trace metals or bind low affinity sites, leading to protein dysfunction, which organisms require for homeostatic mechanisms to control the levels and distribution of this essential metal (Fosmire, 1990). Zinc toxicity is believed to inhibit Calcium uptake, resulting in decreased plasma Calcium concentrations, followed by hypocalcemia (Spry & Wood, 1984; Spry & Wood, 1985).

Previous work has established that the bioaccumulation of silver is significantly influenced by the presence of Zinc, Copper and Se in the water, the action of these three

elements producing an increase in the amounts of Silver in the organisms (Watts, 1989). Watts (1989) revealed that it should also be noted that excessive intake of Zinc over prolonged periods can also produce a deficiency of Chromium, Copper, Iron and Manganese. Related studies have also suggested an antagonistic relationship between Zinc and Phosphorus (Shivay & Kumar, 2005).

Table 11: A list of identified elemental composition measured by ICP−MS of nematodes exposed to Zinc. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. ICP−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Zn 340 µM		Zn 679 µM		Zn 1019 µM		Zn 1358 µM	
Ag	↑	Ag	↑	Ag	↑	Ag	↑
Cd	↓	Bi	↓	As	↓	Al	↓
Ce	↓	Cd	↓	Ba	↓	As	↓
Se	↓	Cs	↓	Bi	↓	Ba	↓
Mg	↓	Fe	↓	Cd	↓	Bi	↓
Mn	↓	S	↓	Cr	↓	Ca	↓
		Se	↓	Cs	↓	Cd	↓
		Mg	↓	Dy	↓	Cr	↓
		Mn	↓	Er	↓	Cs	↓
		Ni	↓	Ag	↓	Dy	↓
				Fe	↓	Er	↓
				Ga	↓	Fe	↓
				Li	↓	Ga	↓
				Lu	↓	Gd	↓
				Mg	↓	La	↓
				Mn	↓	Li	↓
				Mo	↓	Lu	↓
				Ni	↓	Mn	↓
				P	↑	Mo	↓
				S	↓	Nb	↓
				Se	↓	Ni	↓
				Sr	↓	P	↑
				Tb	↓	Pr	↓
				U	↓	S	↓
				Yb	↓	Se	↓
						Sm	↓
						Sr	↓
						Tb	↓
						Ti	↓
						Tm	↓
						U	↓
						Y	↓
						Yb	↓

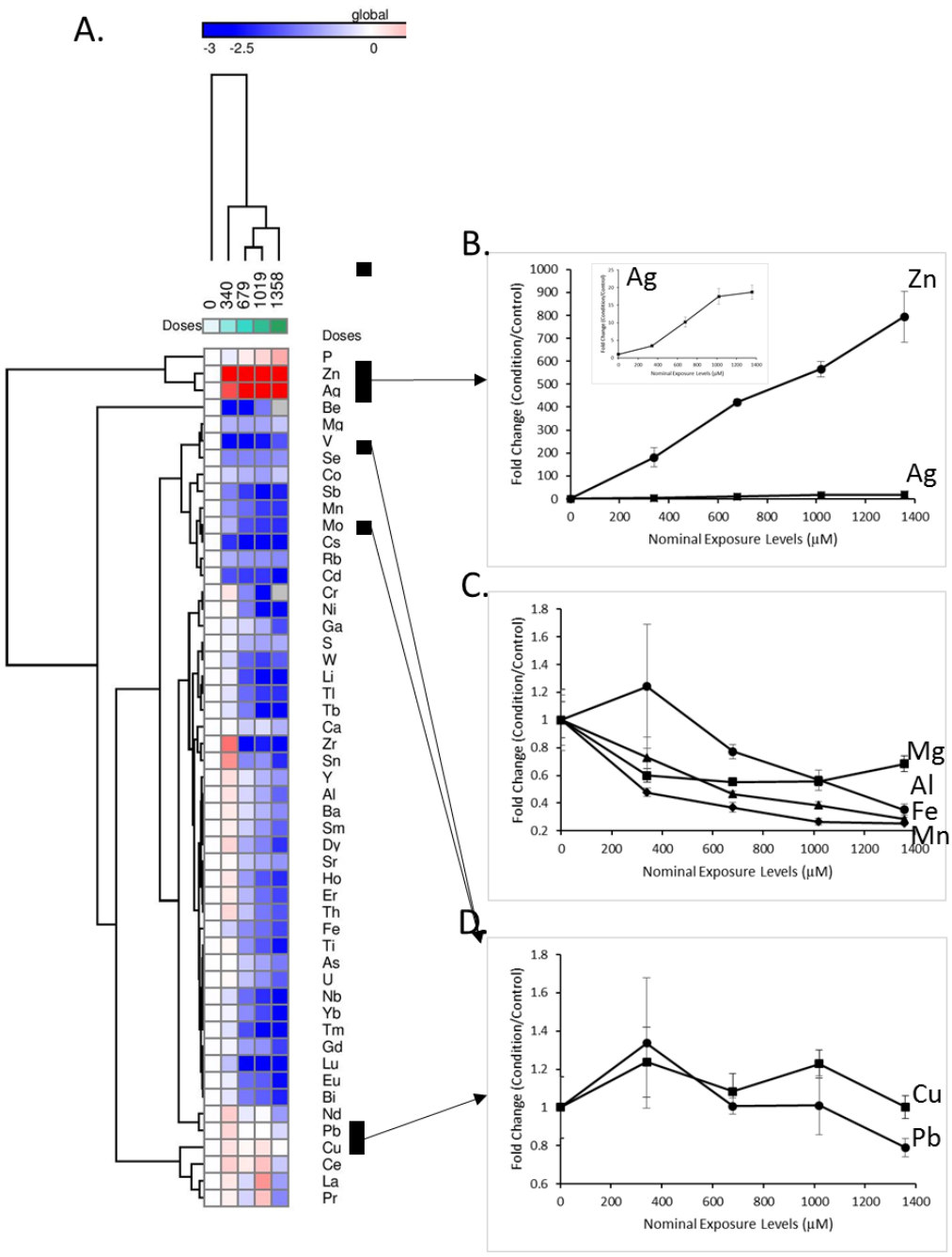

Figure 29: Changes in elemental composition measured by ICP-MS of nematodes exposed to Zinc. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. **Panel A:** Hierarchical clustering of changes in ions levels in response to Zinc exposure. Log_2 (Mean fold change) is clustered for ion and condition using a minus one Pearson correlation with average linage method applied. **Panels B-C:** Example individual metal ion fold changes in response to Zinc exposure. All points are mean with error bars indicating ± SE.

5.2.3.4 SYSTEMS LEVEL ANALYSIS OF ZINC IMPACT

In order to derive the relationships between the impact of Zinc at different level of biological organisation and determine the molecular (metabolite or ion) content having the most significant influence on life history traits, we constructed linear models to predict continuous linear relationships between the Zinc exposure (target) and one or more molecular/life history parameters (predictors).

According to the SPSS Automatic Linear Modeling, we may categorizing the top 6 important predictors in tow main classes (Positive and Negative) that related to the different Zinc exposures depending on the estimating coefficient as follow:

- Positive Predictors: C13:0, Zinc and C20:5n3.
- Negative Predictors: C21:0, Serine and C18:0.

Comparing results from the Automatic Linear Modeling (SPSS) with more established statistic tests (ANOVA and t-test), we could reject some of the predictors suggested by the analysis. C18:0 was this parameter and was found to have been increased with Zinc exposures.

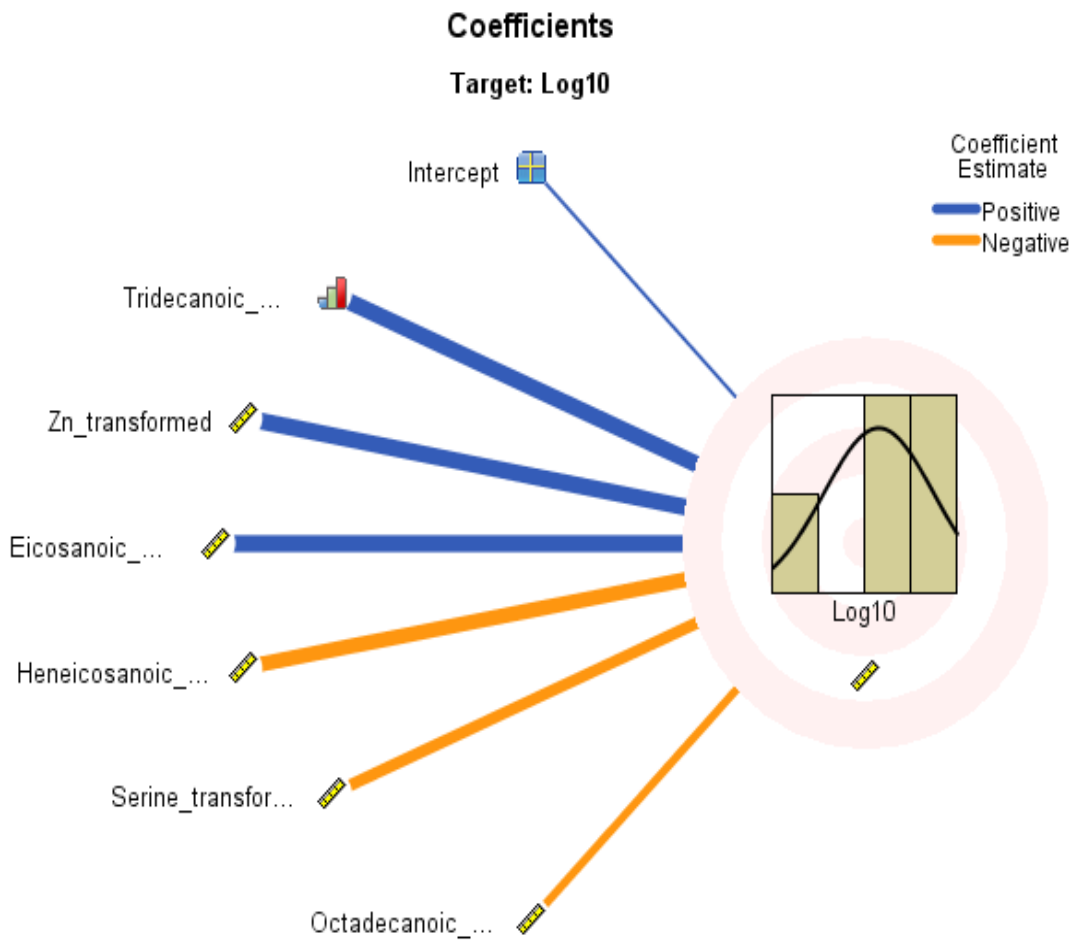

Figure 30: Coefficients (SPSS Linear Models) for Zinc exposure and most important effects. This chart which displays the intercept first, and then sorts effects from top to bottom by decreasing predictor importance. Within effects containing factors, coefficients are sorted by ascending order of data values. Connecting lines in the diagram are coloured based on the sign of the coefficient (Positive or Negative) and weighted based on coefficient significance, with greater line width corresponding to more significant coefficients (smaller *p*-values). The target is Zinc exposure concentrations (Log 10).

5.3 DISCUSSION

Our findings have showed that Zinc has a remarkable effect on the nematode fatty acid profile, decreasing the amount of all fatty acids except the C18:0 which increased in amount when compared with control samples. The presence of fatty acid abnormalities in *Acrodermatitis enteropathica*, a hereditary Zinc absorption disease, and in transient Zinc deficiencies was observed (Brown and Peerson, 2002). Increased level of linoleic acid (18:2n-6) and decreased arachidonic acid (20:4n-6) support the theory that Zinc plays a part in the linoleic acid (18:2n-6) metabolism but the nature of this role has not been identified yet. It is likely to be the result of an explicit variation of the desaturase activities involved in the fatty acid metabolism and a subsidiary effect by influencing absorption, oxidation and incorporation of the fatty acids. It was suggested by Cunnane and Krieger (1988) that Zinc plays a role in the desaturation of linoleic acid (18:2n-6), based on increased linoleic acid (18:2n-6) and decreased (20:4n-6) concentrations in various tissues of the Zn-deficient rats (Hurley and Horrobin, 1985). This postulate was strengthened when decreased Δ-5 and Δ-6 desaturase activity in various tissues of Zn-deficient rats was observed (Cunnane *et al.*, 1984) but could not be corroborated by other experiments (Kramer *et al.,* 1996; Eder and Kirchgessner, 1996). No changes or increase in other desaturase metabolites such as eicosapentaenoic acid (20:5n-3), 22:4n-6 and 22:5n-6 was observed (Eder and Kirchgessner, 1996; Eder *et al.,* 1994). All these findings stand against the direct influence on desaturase activity. Unexpected and opposite effects of Zinc deficiency were noted in different tissues. For instance, Cunnane and Wahle (1981) noted an increase in the Δ-6 desaturation in the mammary tissue of Zinc deficient rats. A connection between serum Zinc and fatty acids in Cystic Fibrosis (CF) patients was identified by (Hamilton *et al.,* 1981). He distinguished a positive relation between serum Zinc and (20:4n-6), 20:3n-6 and the (20:4n-6) / (18:2n-6) ratio. It was further

suggested that optimization of the Zinc status may improve the fatty acids profiles of the CF patients and decreases the inflammatory state (Biervliet, 2008).

Our Ionomics results showed that Zinc exposure had a significant impact on internal elemental balance in *C. elegans*. There was a gradual increase in body burden with increasing exposure indicated by a 2:1 relationship between body burden and exposure. The secondary impacts of Zinc exposure include increasing amounts of Silver and Phosphorus; however, the majority of the remaining ions decreased. Also, we have found that Zinc has reduced Calcium and Nickle. It has previously been postulated that Zinc toxicity inhibits Calcium uptake, resulting in decreased plasma Calcium concentrations and followed by hypocalcemia (Santore *et al.,* 2002). Nickle also exhibits toxicity by blocking several different Calcium channels and disrupting Calcium homeostasis. The specific mechanism of Nickle toxicity to fathead minnows has not yet been elucidated (Macomber & Hausinger, 2011). Cadmium acts as a Calcium mimic due to its similar chemical properties, including ionic radius (Lee *et al.,* 1999; Moulis, 2010). It is considered that Calcium intake is obstructed by Zincn toxicity that results in decreased plasma Calcium concentrations leading to hypocalcaemia (Santore *et al.,* 2002). The nature, however, of Nickle toxicity and its effects on fathead minnows are topics that still need clarity. In the opinion of Ribeyre *et al.,* (1995), bioaccumulation of silver is greatly affected by the amount of Zinc, Copper, and Selenium present in water, the combined action of the elements responsible for increased amounts of Silver in the organisms. It was pointed out by Watts (1988) that large amount of Zinc intake over extended time periods led to a deficiency of Chromium, Copper, Iron, or Manganese. An incompatible relationship between Zinc and Phosphorus was identified by Das *et al.*, (2005), relative to their quantities in soil and contents in plants. Shivay and Kumar (2005), compiled the results of many controlled greenhouse studies to show that intake of Phosphorus is affected by Zinc deficiency and consequently plants that amass Phosphorus

display symptoms of Zinc deficiency. Intriguingly in our studies the increased Zinc levels are mirrored by subtle accumulation of Phosphorus Figure 33 illustrated the complex inter-relatedness the cellular levels of differential essential ions. It will be essential to contrast how the intricate balance of ions are changed by each of our test metals before considering the impact of the metal mixtures.

6 SUB-LETHAL TOXICITY ASSESSMENT OF ALUMINIUM IN *CAENORHABDITIS ELEGANS*

6.1 INTRODUCTION

Aluminium (Al) is the most abundant crustal metal. Although Aluminium is largely insoluble at neutral pH, its bioavailability increases sharply with decreasing pH. Studies of Aluminium toxicity in humans have suggested a link between Aluminium exposure and some forms of microcytic anaemia, osteomalacia and dementia (McNamara, 2001). While the molecular mechanisms underlying these disorders are generally unknown, it has been suggested that elevated Aluminium may be the cause of the disruption of metallostasis, and that this is achieved through changes in the levels of several key elements, namely Copper (Cu), Calcium (Ca), Sodium (Na), Phosphorus (P), Iron (Fe), Fluorine (F) and Sulphur (S) (Yang, 1998) (Barabasz *et al.*, 2002; Nayak & Chatterjee, 2001; WHO, 1996). Aluminium toxicity may result from the fact that Aluminium has higher binding affinities compared to the physiological binding partners, including the replacement of Mg^{2+} and Fe^{3+} by Al^{3+} in key metallo-enzyme (Yang, 1998).

Detailed biochemical studies have indicated that aluminium exerts strong influence the activity of many enzymes. Aluminium forms tight complexes with ATP so it is a strong inhibitor of numerous enzymes utilising ATP as a substrate, such as Na^+/K^+ ATPase. The following enzymes are also inhibited by aluminium: hexokinase, alkalic phosphatase, choline acetyltransferase, ferrooxidase (Zaman *et al.*, 1993; Barabasz *et al.*, 2002). Aluminium ions also show affinity for DNA and RNA (Barabasz *et al.*, 2002). Barabasz *et al.*, (2002) also observed that in the cells, Aluminium is located mainly in the nucleus, where it binds to chromatin, and in lysosomes in equimolar combinations with phosphate groups. Moreover, Aluminium was shown to inhibit important

neurotransmitters, isolated from synapses (γ-aminobutyric acid, 1-glutamate, choline, noradrenaline and serotonin) which play a role in the transmission of nervous impulses. Aluminium is also responsible for nerve fibre degeneration, leading to a decreased number of microchannels in the damaged neurones, dendrites and the cells implicated in memory processes. In other animals, e.g. birds and mammals, Aluminium is usually taken in with food and evokes diversified toxic actions. Toxicity of Al^{3+} to fish is caused by precipitation of Al^{3+} on the gill as the pH is raised in water passing the gill. In birds, Aluminium most frequently affects egg shells and the metabolism of Calcium and Phosphorus, causing diminished efficacy of Calcium absorption and decreased metabolic rates of its transformations, resulting in Aluminium incorporation into bones (Barabasz *et al.,* 2002).

As an element, Aluminium is always found attached to other chemicals, and these affinities can change within the body. In living organisms, Aluminium is believed to exist in four different forms: as free ions, as low-molecular-weight complexes, as physically bound macromolecular complexes, and as covalently bound macromolecular complexes (Ganrot, 1986). The free ion, Al^{3+}, is easily bound to many substances and structures; therefore, its fate is determined by its affinity to each of the ligands and their relative amounts and metabolism. Aluminium may also form low-molecular-weight complexes with organic acids, amino acids, nucleotides, phosphates, and carbohydrates. These low-molecular-weight complexes are often chelates and may be very stable. The complexes are metabolically active, particularly the nonpolar ones. Because Aluminium has a very high affinity for proteins, polynucleotides, and glycosaminoglycans, much of the Aluminium in the body may exist as physically bound macromolecular complexes with these substances. Metabolically, these macromolecular complexes would be expected to be much less active than the smaller, low-molecular-weight complexes. Aluminium may also form complexes with macromolecules that are so stable that they are essentially

irreversible. For example, evidence suggests that the nucleus and chromatin are often sites of Aluminium binding in cells (ATSDR, 2001).

Due to the exceptionally low solubility of Aluminium in water, its content in water bodies is very low, ranging from 60 to 300 µg/L at pH 5-9. In river water, it averages 64 µg/L in sea water it is about 1-5 µg/L, while in water of open oceans it amounts to only 0.5 µg/L (Barabasz *et al.,* 2002). Increased acid rainfalls have caused acidification of surface waters in many areas, which in turn have resulted in mobile Aluminium release from metastable compounds in bottom sediments and their transition into water. It is intriguing that we observed a significant Aluminium within the gill and liver of the fish caught in the Red Sea (Section 3.2.5) given minimal levels were detected within the water, however, significant levels were identified in related sediments (Section 3.2.3). The current investigations aimed at identifying the possible impact of Aluminium on the chosen model organism, *C. elegans*, to investigate the underlying molecular, ion and metabolite, change that underpin life history impacts of Aluminium.

6.2 RESULTS

6.2.1 MORTALITY

To derive the lethality profile of Aluminium within out test system we performed 24 hours exposure of adult nematodes (See Section 2.4.8) to concentrations of Aluminium estimated from the literature as ranging between the $LC_{10} - LC_{40}$ (Table 5; Chu *et al.,* 2002). To compare the effect of different doses of Aluminium on the mortality of *C. elegans* a one-way ANOVA between subjects was conducted. There was a significant effect of amount of Aluminium on mortality of *C. elegans* at the ($p < 0.05$) level at the

two highest concentrations of 404 and 538 µM. This was confirmed using both *post hoc* comparisons using the Tukey's HSD test and independent sample t-test between mortality in each test exposure against that observed in the controls. These results confirmed that at the Aluminium doses employed had a modest impact on mortality survival rates of ~80% being observed in the two higher concentrations Figure 31. The discrepancy between expected and observed survival rates is explained by differences in the time and temperature used for these observation which was 24 hours and 20^{o}C rather than the 48 hours and 22^{o}C, used when the original LC_{50} was determined (Section 2.4.8). A temporal comparison of mortality has been described in Chapter 7.2.1.

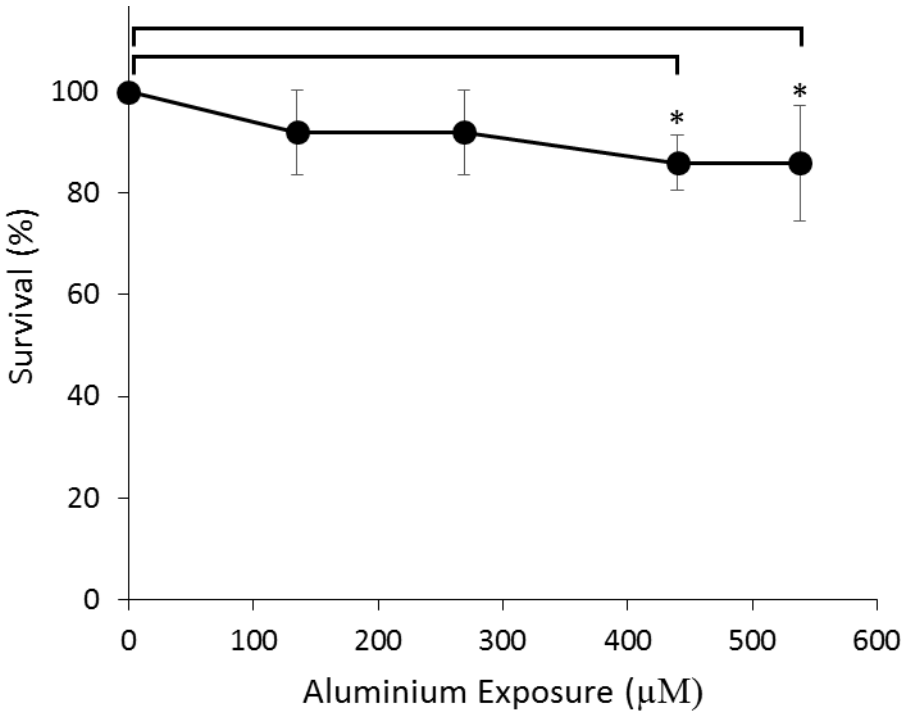

Figure 31: The mortality of *C. elegans* in numbers after Aluminium exposure for 24 hours. 10 adults nematodes from age-synchronized cultures exposed to four different concentrations and used for testing were placed in 1 ml of test solution. The number of survivals was counted. For each test concentration and control, the average number of survivals from the wells was obtained for each trial, and at least five trials were conducted after their exposed with different doses of Aluminium in 24 hours period**.** Points represent mean ± SD. * represents significant compared to the control via t-test ($p < 0.05$).

6.2.2 REPRODUCTION

To evaluate the sub-lethal impact of Aluminium on reproduction we performed a 72 hours exposure using age synchronized cultures (See Section 2.4.9) and employing the identical exposures levels based on previous literature and described above in Section 4.2.1. A comparison of the effect of Aluminium on the number of eggs laid by *C. elegans* (one-way ANOVA) revealed a significant ($p < 0.05$) effect of amount of Aluminium at all four exposure concentrations. This observation was verified using both *post hoc* comparisons using the Tukey's HSD test and independent sample t-test conducted to compare the effect of Aluminium on egg laid number of *C. elegans* between control and each exposure. The lowest exposure levels, Aluminium 135 µM, reduced fecundity to ~30% of controls with subsequent increases resulting in further gradual reductions, with the highest exposure yielding only ~15% of the eggs laid by control nematodes Figure 32. In contrast to our observations, previous studies employing oral-exposure and examining reproduction as an end-point, have suggested that Aluminium was not associated with alterations in fertility (Dixon *et al.*, 2000; ATSDR, 2001).

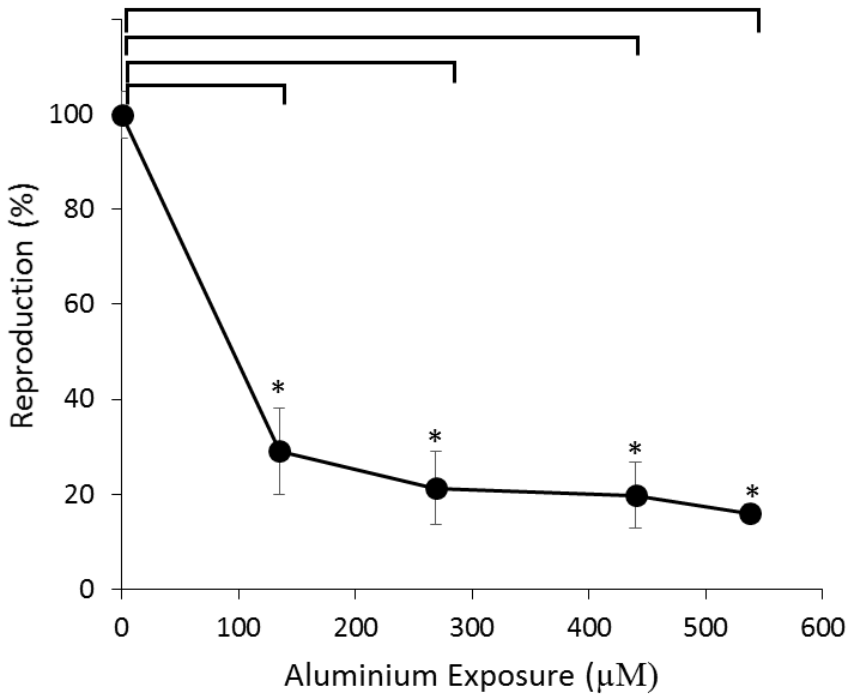

Figure 32: Number of *C. elegans* eggs laid after Aluminium exposure for 72 hours. Single nematodes from age-synchronized cultures exposed to four different concentrations and used for testing were placed in 1 ml of test solution. The number of offspring at all stages beyond the eggs was counted. For each test concentration and control, the average number of progenies from the wells was obtained for each trial, and at least five trials were conducted. Bars represent mean ± SD. * represent significant compared to the control via t-test ($p < 0.05$).

6.2.3 METABOLOMICS

6.2.3.1 AMINO ACIDS

Our results shown Table 12 and Figure 33 provided a global insight into how Aluminium has impacts on the level of hydrophobic metabolites (amino acids) in *C. elegans*. At the lower dose, 135 µM, the level of many amino acid constituents was significantly reduced Table 12 with the exception of Aspartic acid, Hydroxylysine, Ornithine and Tryptophan. Significant reductions in a range of amino acids were also seen when nematodes were exposed to 269 and 404 µM Aluminium respectively, but this impact was reversed at 538 µM, where many of the same amino acids were elevated.

Table 12: A list of differentially regulated aqueous phase metabolites observed in nematodes after Aluminium exposure. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Al 135 µM		**Al 269 µM**		**Al 404 µM**		**Al 538 µM**	
AABA	↓	Allo Isoleucine	↓	Glycine	↓	Glycine	↑
Leucine	↓	Isoleucine	↓	Threonine	↓	Threonine	↑
Allo Isoleucine	↓	Proline	↓	Glutamic	↑	Serine	↑
Isoleucine	↓	Methionine	↓	AAPA	↓	Methionine	↑
Asparagine	↓	Hydroxyproline	↓	Ornithine	↓	Hydroxyproline	↑
Aspartic acid	↑	AAPA	↓	Lysine	↓	Glutamic acid	↑
Methionine	↓	Ornithine	↓	Histidine	↓	Glycine Proline	↑
Hydroxyproline	↓	Lysine	↓	Tryptophan	↓	Cystathionine	↑
AAPA	↓	Cystathionine	↓	Cystathionine	↓	Cysteine	↑
Glutamine	↓			Cysteine	↓		
Ornithine	↑						
Glycine Proline	↓						
Histidine	↓						
Hydroxylysine	↑						
Tyrosine	↓						
Proline	↓						
Tryptophan	↑						
Cystathionine	↓						
Cysteine	↓						

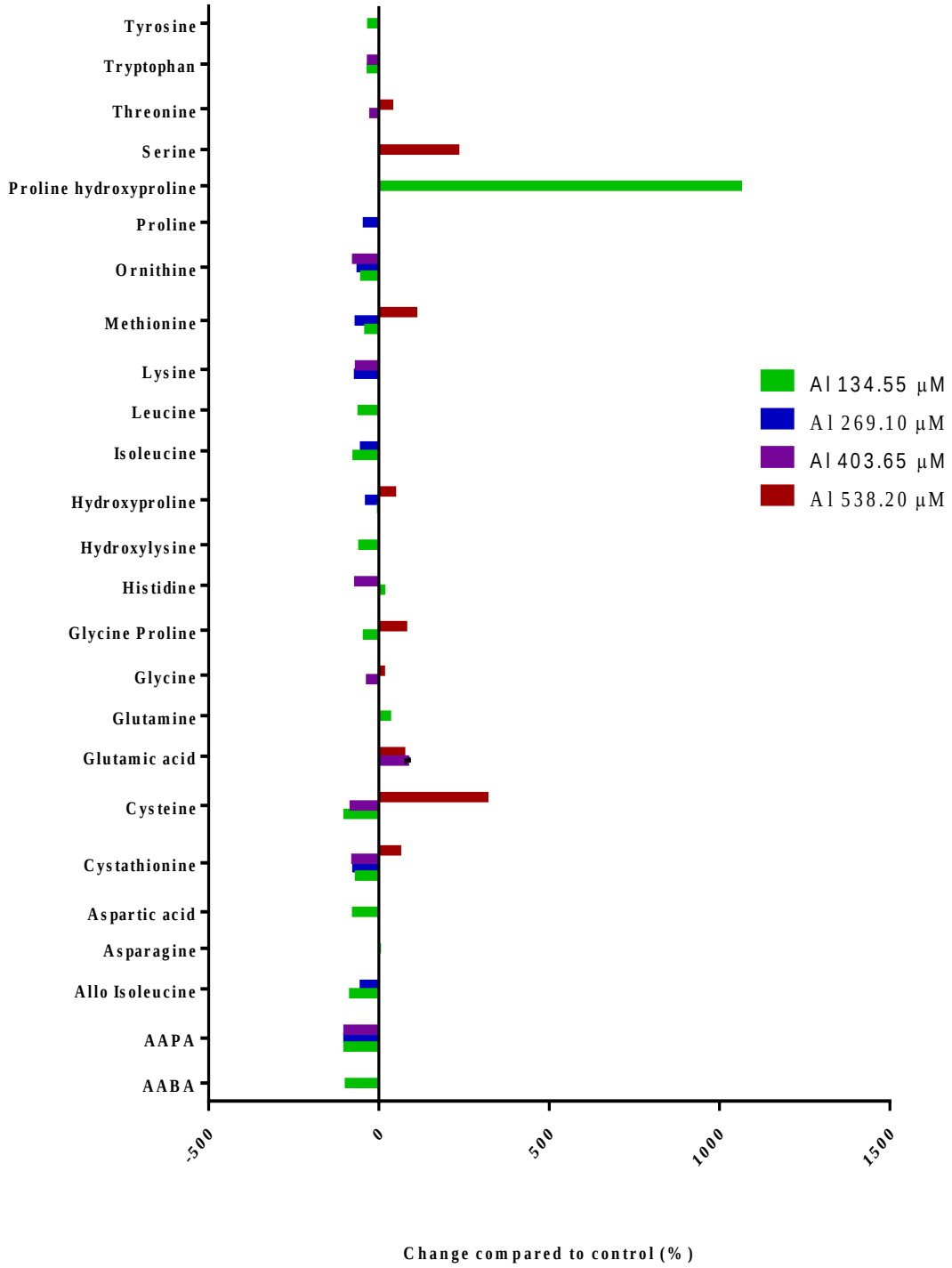

Figure 33: Changes in aqueous phase (Amino Acids) concentrations of Al adult nematodes exposed measured by ICP-MS. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 h period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance after different Aluminium dose exposures for 24 hours. All bars mean percentage change from control ± SD in the chart and each point represents significant difference when compared to control levels ($p < 0.05$).

6.2.3.2 FATTY ACIDS

Our experiments revealed in Table 13 and Figure 34 that Aluminium caused a general decrease in most fatty acids with the exception of C14:0 and C20:4n3, which were observed to be increased under the lowest Aluminium exposure. However, several studies have shown that oxidative stress induced by Aluminium modified the peroxidation of lipids and the activities of anti-oxidative enzymes and identified increased peroxidation of lipids in brain tissues of adult *Wistar* rats following administration of Aluminium intakes (Zaman *et al.,* 1993; Becaria *et al.,* 2002; Ondreička *et al.,* 1966).

Table 13: A list of differentially regulated organic phase metabolites observed in adult nematodes after Aluminium exposure. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Al 135 µM		**Al 269 µM**		**Al 404 µM**		**Al 538 µM**	
C14:0	↑	C15:0	↓	C15:0	↓	C15:0	↓
C15:0	↓	C17:0	↓	C17:0	↓	C17:0	↓
C17:0	↓	C18:2n6	↓	C18:2n6	↓	C18:2n6	↓
C18:2n6	↓	C20:0	↓	C20:0	↓	C20:0	↓
C20:0	↓	C20:4n6	↓	C20:4n6	↓	C20:4n6	↓
C20:4n6	↓						
C20:4n3	↑						

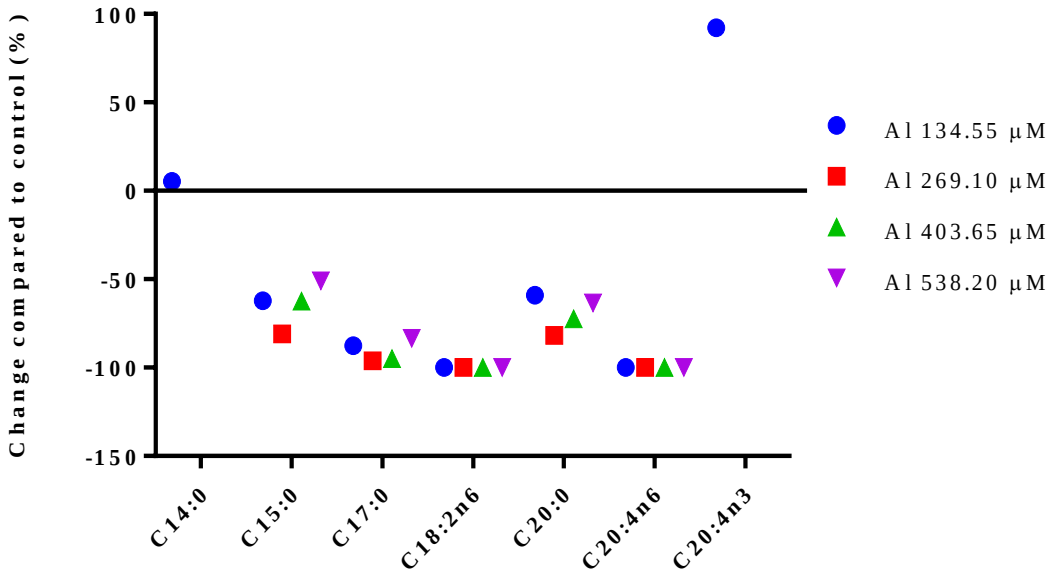

Figure 34: Changes in Fatty Acids composition measured by GC-MS of Aluminium exposed nematodes. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. GC−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance after different Aluminium dose exposures for 24 hours. All points mean percentage change from control ± SD in the chart and each point represents significant difference when compared to control levels ($p < 0.05$).

6.2.4 IONOMICS

Our results Table 14 and Figure 35 have shown that Aluminium causes changes to elemental profiles of whole nematodes. For the two lowest Aluminium concentration, we found large, significant increases in multiple metals known to cause oxidative stress, e.g. Ag, Co, Dy, Er, Eu, Fe, Gd, Ho, La, Nd, P, Pr, Rb, Se, Sm, Sn, Tb, Th, Tl, Tm, U, W and Y. We hypothesized that the accumulation of Aluminium inside the nematodes caused oxidative stress. The two highest Aluminium doses also caused decreased elemental profiles Table 14.

A number of studies have been carried out for observing the effects of different elements on human body. A similar study was carried out for observing the effects of exposure of Aluminium on human body. The Aluminium has higher binding attractions in comparison to physical binding partners and it is for this reason that describes why Aluminium interrupts the physical balance of Iron and Calcium level along with some metals. However, though research has been determined that there were some connections between Aluminium exposure, kinds of dementia, osteomalacia, and microcytic anaemia (Becaria *et al.*, 2002). The molecular mechanisms under such disorders are undefined. Aluminium exposure gives rise to numerous pathologies and since it is already proven to alter the levels of metals in multiple species, we theorized that Aluminium alters global metal homeostasis (Page *et al.*, 2012).

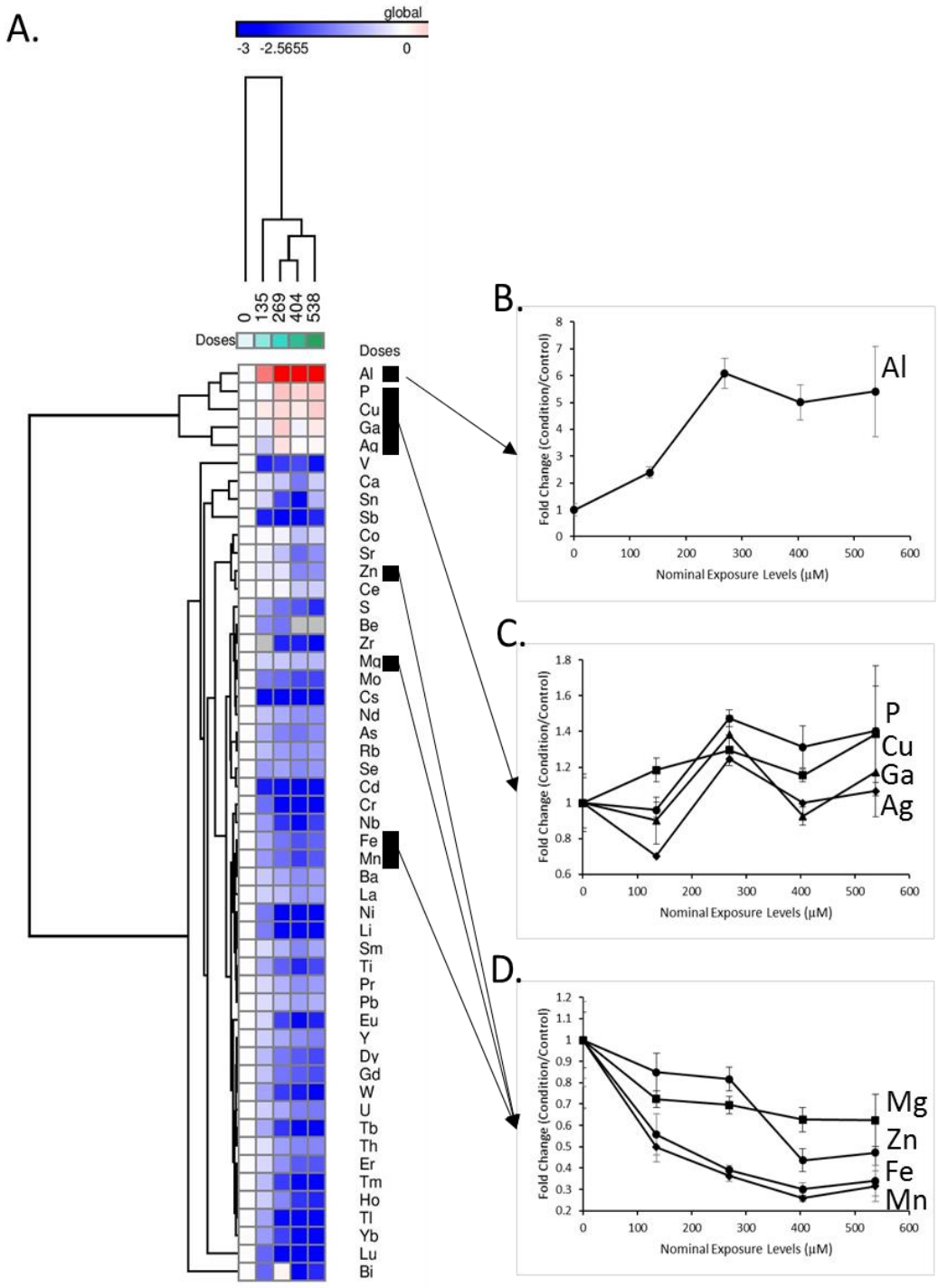

Figure 35: Changes in elemental composition measured by ICP-MS of Aluminium exposed nematodes. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 hours period. Panel A: Hierarchical clustering of changes in ions levels in response to Aluminium exposure. Log_2 (Mean fold change) is clustered for ion and condition using a minus one Pearson correlation with average linage method applied. Panels B-C: Example individual metal ion fold changes in response to Aluminium exposure. All points are mean with error bars indicating ± SE.

Table 14: A list of identified elemental composition measured by ICP−MS of adults nematodes Aluminium exposed. Nematodes were from age-synchronized cultures exposed to four different concentrations and controls. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 h period. ICP−MS data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample for the significant values.

Al 135 µM		**Al 269 µM**		**Al 404 µM**		**Al 538 µM**	
Ag	↓	As	↓	As	↓	As	↓
Be	↓	Ba	↓	Ba	↓	Ba	↓
Bi	↓	Cd	↓	Bi	↓	Bi	↓
Cd	↓	Cr	↓	Ca	↓	Cd	↓
Co	↓	Cs	↓	Cd	↓	Cr	↓
Cs	↓	Dy	↓	Cr	↓	Cs	↓
Dy	↓	Fe	↓	Cs	↓	Dy	↓
Er	↓	Li	↓	Dy	↓	Er	↓
Eu	↓	Mn	↓	Er	↓	Fe	↓
Ga	↓	Ni	↓	Fe	↓	Gd	↓
Gd	↓	P	↑	Gd	↓	La	↓
Ho	↓	S	↓	La	↓	Li	↓
La	↓	Se	↓	Li	↓	Lu	↓
Lu	↓	Y	↓	Lu	↓	Mn	↓
Mo	↓	Yb	↓	Mg	↓	Mo	↓
Nb	↓			Mn	↓	Ni	↓
Nd	↓			Mo	↓	S	↓
Ni	↓			Ni	↓	Se	↓
Pr	↓			Pb	↓	Sr	↓
Rb	↓			Pr	↓	Tb	↓
Sb	↓			S	↓	Ti	↓
Se	↓			Se	↓	Tm	↓
Sm	↓			Sr	↓	U	↓
Sn	↓			Tb	↓	W	↓
Tb	↓			Ti	↓	Y	↓
Th	↓			Tm	↓	Yb	↓
Tl	↓			U	↓		
Tm	↓			Y	↓		
U	↓			Yb	↓		
V	↓						
W	↓						

6.2.5 SYSTEMS LEVEL ANALYSIS OF ALUMINIUM IMPACT

According to the SPSS Automatic Linear Modeling, we may categorizing the top 10 important predictors in tow main classes (Positive and Negative) that related to the different Aluminium exposures depending on the estimating coefficient as follow:

- Positive Predictors: C13:0, Allo-Isoleucine, Cystathionine, Glutamic Acid and Aspartic Acid.
- Negative Predictors: Reproduction, Ornithine, Asparagine, Glycine and C20:5n3.

Comparing results from the new SPSS feature (Automatic Linear Modeling) with the most acceptable statistic tests (ANOVA and t-test), we could reject some of the predictors that do not match such as: Allo-Isoleucine, Cystathionine as this parameters was decrease with Aluminium exposures, while Ornithine was increase with Aluminium exposures.

In order to derive the relationships between the impact of Aluminium at different level of biological organisation and determine the molecular (metabolite or ion) content having the most significant influence on life history traits, we constructed linear models to predict continuous linear relationships between the Aluminium exposure (target) and one or more molecular/life history parameters (predictors).

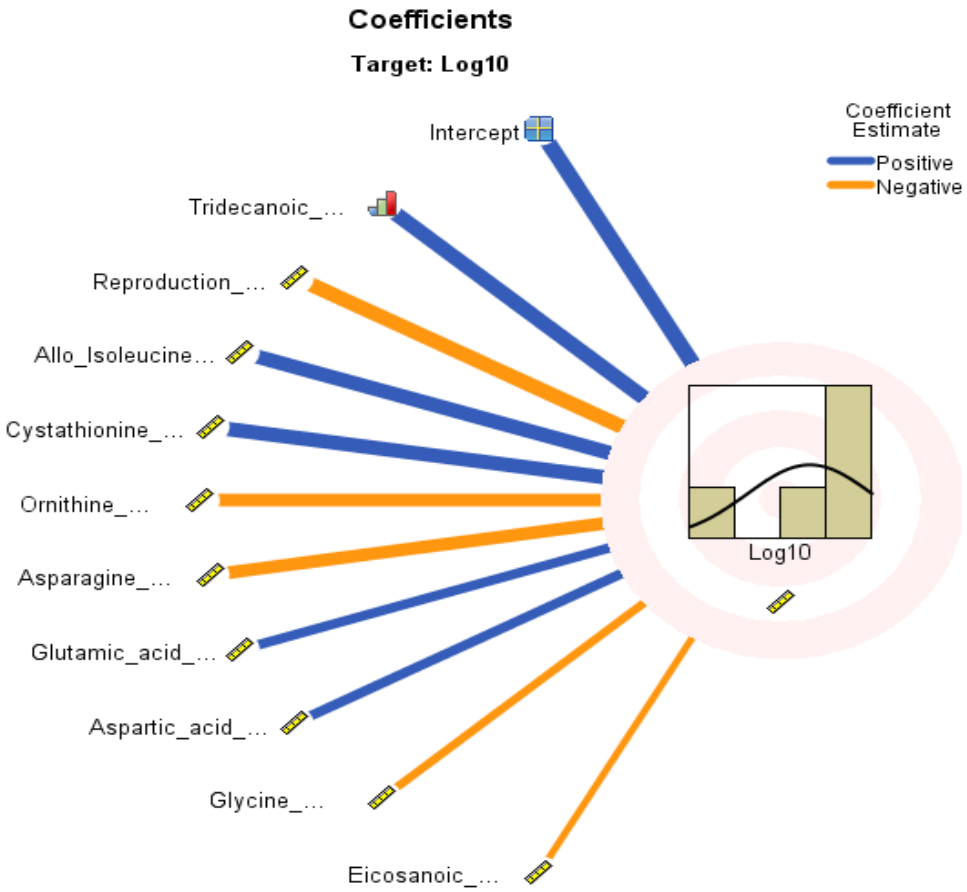

Figure 36: Coefficients (SPSS Linear Models) for Aluminium exposure and most important effects. This chart which displays the intercept first, and then sorts effects from top to bottom by decreasing predictor importance. Within effects containing factors, coefficients are sorted by ascending order of data values. Connecting lines in the diagram are coloured based on the sign of the coefficient (Positive or Negative) and weighted based on coefficient significance, with greater line width corresponding to more significant coefficients (smaller *p*-values).

6.3 DISCUSSION

There was a significant effect ($p < 0.05$) of Aluminium on survival of *C. elegans* at the two highest concentrations of 404 and 538 µM. This was confirmed using both *post hoc* comparisons using the Tukey's HSD test and independent sample t-test between mortality in each test exposure against that observed in the controls. These results confirmed that at the doses employed, Aluminium had a modest impact on mortality with survival rates of ~80% being observed in the two higher concentrations.

A comparison of the effect of Aluminium on the number of eggs laid by *C. elegans* revealed a significant effect ($p < 0.05$) of Aluminium at all four exposure concentrations. This observation was verified using both *post hoc* comparisons using the Tukey's HSD test and independent sample t-test conducted to compare the effect of Aluminium on numbers of eggs laid by *C. elegans* between control and each exposure. The lowest Aluminium exposure levels (135 µM) reduced fecundity to ~30% of controls with subsequent increases resulting in further gradual reductions with the highest exposure yielding only ~15% of the eggs laid by control nematodes. No studies have commented on the reproductive effects of various forms of Aluminium following inhalation, oral or dermal exposure in humans (ATSDR, 2001). No histological alterations were observed in the reproductive tissues of rats or guinea pigs exposed to airborne Aluminium chlorohydrate (Steinhagen *et al.,* 1978). In general, the results of these studies have been conducted on *Albion* rats, suggesting, that Aluminium was not associated with alterations in fertility (Dixon *et al.,* 2000). In our study, Aluminium exposure clearly reduced fertility. We counted the egg laying production of nematodes exposed to Aluminium compared to the control sample and the Aluminium significantly reduced the total number of eggs.

Our results have provided us with a global insight into how Aluminium has impacts on the level of hydrophobic metabolites (amino acids) in *C. elegans*. At the lower dose (135 µM), many of the amino acid constituents were significantly reduced with the exception of Aspartic acid, Hydroxylysine, Ornithine and Tryptophan. Significant reductions in a range of amino acids was also observed when nematodes were exposed to 269 and 404 µM, Aluminium but interestingly this impact is reversed at 538 µM where many of the same amino acids were shown increase. Nayak & Chatterjee (2001), reported in their study that the glutamate level was noted to be increased significantly in all the tested brain regions of Aluminium-treated rats. One of the contributory factors for this increase in glutamate level may be Aluminium-induced inhibition of glutamate transport by the synaptic vesicles or the inhibition of Glutamate release by Aluminium. Barabasz *et al.,* (2002) noted that Aluminium in cells was found primarily in the nucleus attached to chromatin, as well as in equimolar quantities attached to phosphate groups in lysosomes. Aluminium also has a tendency to hinder critical neurotransmitters, excluded from synapses (γ-amino butyric acid, Glutamate, Choline, Noradrenaline and Serotonin) which play a part in the transmission of nervous impulses. Another type of damage caused by Aluminium is the degeneration of nerve fibres and a decreased number of micro-channels in already damaged neurones, dendrites and the cells associated with memory processes. Nayak and Chatterjee (2001), found excessively high glutamate levels in his testing of brain region for Aluminium exposed rats. This increase in glutamate level can also be caused by aluminium-induced inhibition of glutamate transport by the synaptic vesicles or the inhibition of Glutamate release by Aluminium. Glutamate levels in the brain may also be tempered by its changed metabolism. The modified metabolism of glutamate caused by Aluminium has brought about several Glutamate metabolizing enzymes leads to fluctuations in its concentration.

Our fatty acids experiments showed an expected result with Aluminum, as it has caused a general decrease in most of the fatty acids measured with the exception of C14:0 and C20:4n3, which were observed to be increased under the lowest Aluminium exposure. However, several studies have shown that oxidative stress induced by Aluminium modifies the peroxidation of lipids and the activities of anti-oxidative enzymes and identified increased peroxidation of lipids in brain tissues of adult *Wistar* rats following administration of Aluminum intake (Zaman *et al.,* 1993; Becaria *et al.,* 2002; Ondreička *et al.,* 1966).

Our results have shown that Aluminium causes changes to elemental profiles of whole nematodes. For the two lowest Aluminium concentration. We hypothesized that the accumulation of Aluminium inside the nematodes caused oxidative stress. However, the two highest Aluminium doses also caused decreased in elemental profiles. In cases of very high Aluminium concentrations, large increases were observed in multiple metals known to cause oxidative stress, e.g. Barium, Iron, Chromium, and Copper. It is hypotheses that accumulation of Aluminium inside the nematodes causes oxidative stress and consequently results in Al-exposure phenotypes such as; reproduction, metabolomics and metallostasis. Aluminium exposure gives rise to numerous pathologies and it is already proven to alter the levels of metals in multiple species. It has been theorized that Aluminium alters global metal homeostasis (Page *et al.,* 2012). It was shown by Yang *et al.,* (1998) that chronic exposure to $AlCl_3$ increased Aluminium levels in the mouse brain, reduced Copper concentrations and increased Zinc content. According to these authors (Page *et al.,* 2012; Yang *et al.,* 1998), this high level of Zinc could be a likely cause of Al-stimulated dementia (Page *et al.,* 2012; Yang *et al.,* 1998). It is our proposition that high levels of Aluminium influence metallostasis by causing changes in the trace element pool and that this disturbance results in an inequality in elemental concentrations and stimulates the production of oxidative stress. For example, in our results showed that high

doses of Aluminium has caused reduction in Copper and Zinc concentrations and these metals are known as responsible for SOD activity that is working as metal detoxification system.

7 COMPARISONS AND CORRELATIONS ENDPOINTS ASSESSMENT OF SINGLE SUB-LETHAL METALS FOR TOXICITY USING *CAENORHABDITIS ELEGANS*

7.1 INTRODUCTION

According to Dupont *et al.,* (2010), a heavy metal, particularly a transition metal such as Copper, Iron, Manganese or Zinc is essentially required as a metal cofactor by approximately 30% of all proteins available. The metal ions as well as proteins are also involved in metal-mediated control of gene expression (Jackson *et al.*, 2008). Metal ion involvement in intracellular signalling may also be either direct (as explained by Yamasaki *et al.,* (2007)), or indirect (as mentioned by Evstatiev & Gasche, (2012)).

Most of the biological processes of living systems require metal ions as one of the basic elements for their efficient functioning. Therefore, tight control of homeostasis and all the associated activities of these metals are also required (Bertini & Cavallaro, 2008). If the metal ion homeostasis is disturbed, the metals can either bind to the undesirable sites or displace the other correctly bound metals from their respective sites (Nelson, 1999). The metal ion transporters are basically involved in maintaining the homeostasis and, hence, the desired levels of metal ions in the body (Rolfs & Hediger, 1999). In the case of transporter failure to regulate metal ions, homeostasis of the metal ions is derailed, which results in development of a large number of diseases that target DNA and other proteins, and ultimately, cause oxidative deterioration of biological macromolecules, as the free radical theory of aging and the roles played by reactive species in signal transduction, cell death, human reproduction, and other important biological events (Halliwell and Gutteridge, 2007).

7.2 RESULTS

7.2.1 MORTALITY

To derive the lethality profile of our tested metals (Copper, Zinc and Aluminium) within out test system, we performed 24 hours exposures of adult nematodes (See Section 2.4.8) to concentrations of our tested metals (Copper, Zinc and Aluminium); pre-estimated from the literature ranging between the $LC_{10} - LC_{40}$ (Table 5; Chu *et al.*, 2002). Comparing the effect of different doses of (Copper, Zinc and Aluminium) on the mortality after 24 hours on *C. elegans* (one-way ANOVA) revealed no significant effect of all the tested metals on the mortality of *C. elegans* ($p < 0.05$). According to the mortality test graph revealing the acute toxicity at different concentrations, the metals were also categorized into two major groups. The mortality percentage for Copper and Aluminium exposure were between 85% - 95%, while the mortality percentage for Zinc in the low concentrations (LC_{10} and LC_{20}) were around 95%, but in high concentrations (LC_{30} and LC_{40}) were below 80% Figure 37. The discrepancy between expected and observed survival rates may be explained by differences in the time and temperature used for these observation (24 hours and 20^{o}C rather than the 48 hours and 22^{o}C used when the original LC_{50} was determined) (Section 2.4.8). A temporal comparison of mortality has been described in Chapter 7.2.1.

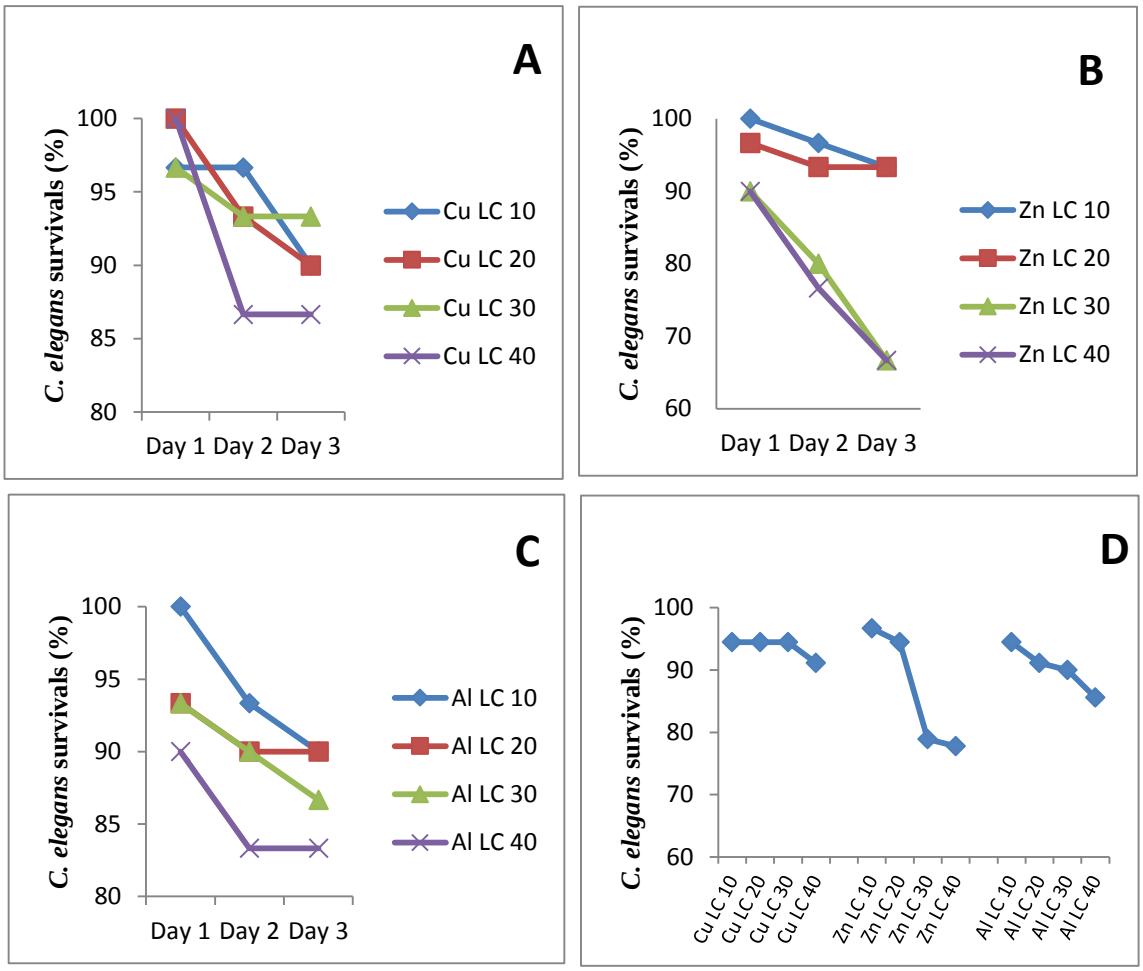

Figure 37: The mortality percentage of *C. elegans* for three different metals. (A) Copper exposure, (B) Zinc doses and (C) Aluminium amounts (D) Comparison of the all exposed metals in different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) in a 3-day exposure experiment.

Comparison between the chosen tests metals (Copper, Zinc and Aluminium) in the same level of the doses related to the exposed period for the mortality was assessed over 72 hours Figure 38 by counting dead versus live nematodes under a dissecting microscope. According to the graph, the mortality percentage for Copper, Zinc and Aluminium in the LC_{10} and the LC_{20} exposures were reduced dramatically by Day 3 (90% - 92%), while the mortality percentage for the metals in LC_{30} Copper and Aluminium

were between 92% - 94% but for Zinc reduced to 80%. In high concentration LC_{40}, Copper and Aluminium were below 90%, while for Zinc, the lowest was at 67%.

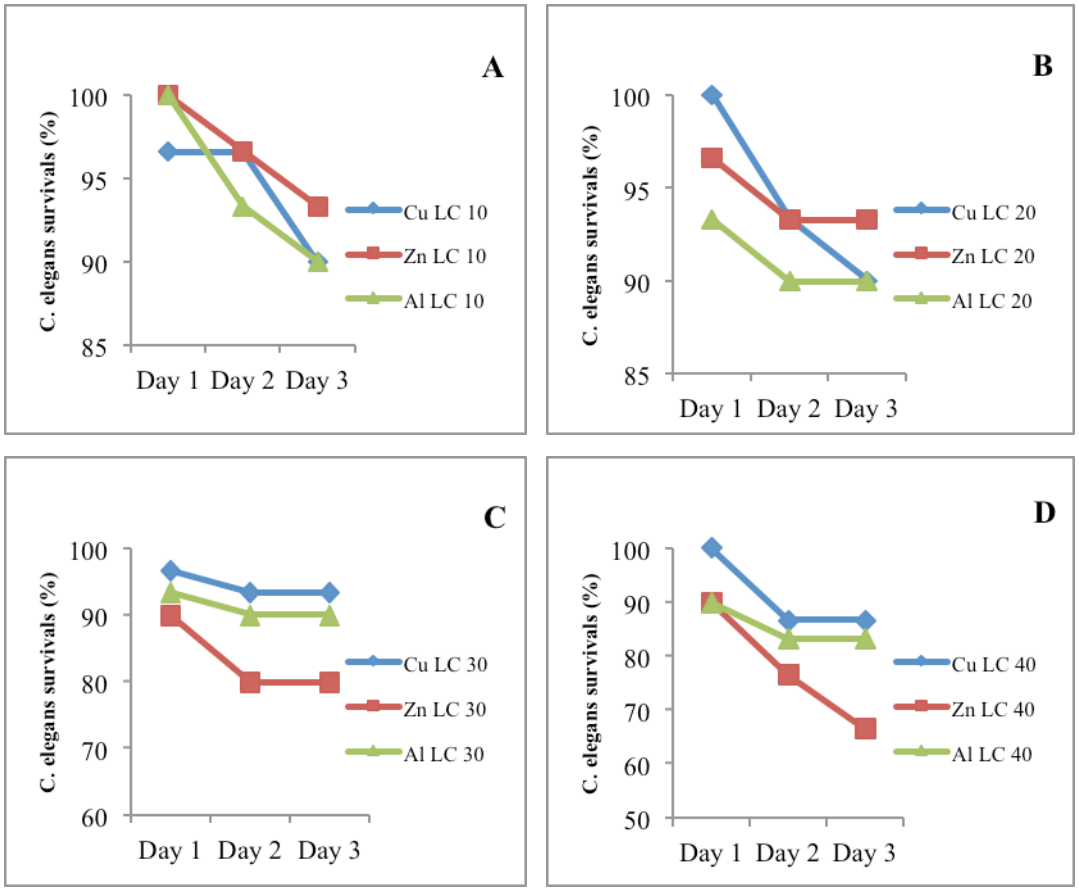

Figure 38: The mortality percentage of *C. elegans* for three different metals continued. (A) Copper exposure, (B) Zinc doses and (C) Aluminium amounts (D) Comparison of the all exposed metals in different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) in a 3-day exposure experiment.

7.2.2 REPRODUCTION

Under optimal conditions, in the absence of the chosen metals (Copper, Zinc and Aluminium), the wild type strain produced 93 ± 5 viable eggs with the expected normality. In general, with increasing metal concentrations, total hatched eggs were reduced Figure 39. For Copper exposure the hatched eggs in LC_{10}, LC_{20} and LC_{30} were produced almost by 64% and got lower in the highest dose LC_{40} to reach ~ 45 hatched

eggs per nematode. Hatched eggs for all Zinc concentrations were slightly stable between 53 – 56 hatched eggs. While in Aluminium exposure, the hatched eggs number were the lowest for all exposed metals. The egg number in LC_{10} was ~ 40 hatched eggs and was reduced 34 eggs in the rest Aluminium doses.

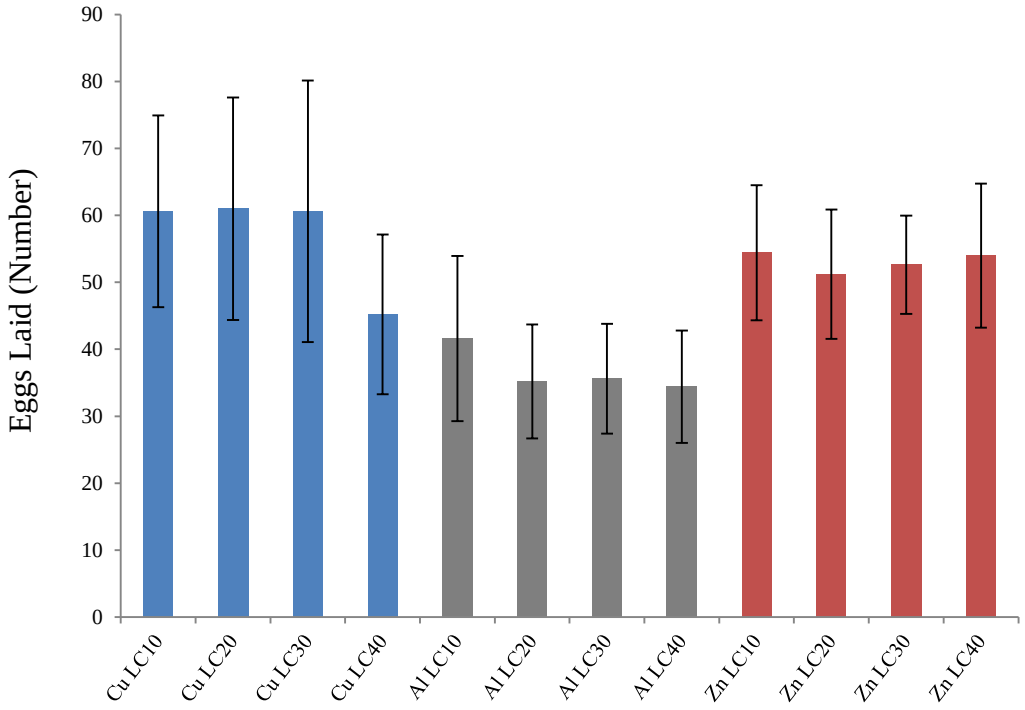

Figure 39: Number of *C. elegans* hatched eggs per nematode after exposure for different metals (Copper, Zinc and Aluminium). In different doses counted after a 3-day exposure experiment. All bars represent the mean average change from control ± SD in the chart and each point represents significant difference when compared to control levels ($p < 0.05$).

7.2.3 METABOLOMICS

7.2.3.1 AMINO ACIDS

It has been shown in the collective results in Table 15 that the two highest sub-lethal Copper caused an elevation in Cystathionine and Cysteine levels, while that effect was not reported in the remaining two lowest doses. Also, it was noticed that for most of Copper concentrations Glycine and Tryptophan had been reduced, while BAIBA and Histidine were increased. For sub-lethal Zinc exposures in *C.elegans* have been generally increased most of the metabolite (amino acids) compositions increased except for BAIBA, Leucine, Isoleucine and Glutamic acid, which decreased. In addition, Aluminium had an impact on some metabolites (amino acids) by dropping their amounts in *C. elegans,* with the exception of Aspartic acid, Hydroxylysine and Glutamic acid, which increased. Furthermore, the highest dose caused an increase in all of the Amino acids detected.

Table 15: A list of identified aqueous phase metabolites (Amino Acids) measured by GC−MS. Data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample.

Amino acids	Cu LC_{10}	Cu LC_{20}	Cu LC_{30}	Cu LC_{40}	Zn LC_{10}	Zn LC_{20}	Zn LC_{30}	Zn LC_{40}	Al LC_{10}	Al LC_{20}	Al LC_{30}	Al LC_{40}
AABA				↑					↓			
AADA			↑	↑								
AAPA							↑		↓	↓	↓	
Allo Isoleucine	↓		↑			↑	↓	↑	↓	↓		
Asparagine									↓			
Aspartic acid									↑			
BAIBA	↑	↑	↑		↓			↓				
Cystathionine			↑	↑			↑		↓	↓	↓	↑
Cysteine				↑		↑		↑	↓		↓	↑
Glutamic acid								↓			↑	↑
Glutamine		↓	↓					↑	↓			
Glycine	↓	↓	↓	↓		↑	↓				↓	↑
Glycine Proline	↓	↓				↑			↓			↑
Histidine		↑	↑	↑		↑		↑	↓		↓	
Hydroxylysine									↑			
Hydroxyproline			↑	↑			↑	↑	↓	↓		↑
Isoleucine	↓		↑			↑	↓	↑	↓	↓		

Table 15 (Continued): A list of identified aqueous phase metabolites (Amino Acids) measured by GC−MS. Data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample.

Amino acids	Cu LC_{10}	Cu LC_{20}	Cu LC_{30}	Cu LC_{40}	Zn LC_{10}	Zn LC_{20}	Zn LC_{30}	Zn LC_{40}	Al LC_{10}	Al LC_{20}	Al LC_{30}	Al LC_{40}
Leucine	↓		↑			↑	↓	↑	↓			
Lysine								↑		↓	↓	
Methionine			↑			↑		↑	↓	↓		↑
Ornithine									↑	↓	↓	
Phenylalanine			↑	↑		↑		↑				
Proline						↑				↓		
Proline			↑	↑					↓			
Serine								↑				↑
Threonine		↓	↓			↑		↑			↓	↑
Tryptophan	↓	↓	↓						↑		↓	
Tyrosine			↑	↑		↑		↑	↓			
Valine	↓		↑			↑						

7.2.3.2 FATTY ACIDS

Our findings, demonstrated that Copper had decreased the total fatty acids content in *C. elegans* except, for one fatty acid, which was C18:2n6. Zinc had a remarkable effect on the nematode fatty acids profile, whereby most were reduced in concentration except for C18:0 that increased in concentration (versus controls). Table 16 demonstrated that Aluminium caused decrease in most fatty acids except for C14:0 and C20:4n3 at the lowest Aluminium sub-lethal exposure dose. In general, it has been observed from the comparison Table 16 that Copper, Zinc and Aluminium had a significant impact on fatty acids by decreasing their concentrations in *C. elegans*.

Table 16: A list of identified organic phase metabolites (Fatty Acids) measured by GC−MS. Data after the ANOVA test and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ represent the mean difference of the exposed samples compared to the mean of the control sample.

Fatty acids	Cu LC_{10}	Cu LC_{20}	Cu LC_{30}	Cu LC_{40}	Zn LC_{10}	Zn LC_{20}	Zn LC_{30}	Zn LC_{40}	Al LC_{10}	Al LC_{20}	Al LC_{30}	Al LC_{40}
C22:0		↓	↓									
C20:5n3	↓	↓										
C20:0				↓	↓	↓	↓	↓	↓	↓	↓	↓
C20:4n6	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C20:4n3									↑			
C21:0		↑	↓	↓	↓		↓	↓				
C17:0	↓	↑		↓	↓	↓	↓	↓		↓	↓	↓
C18:2n6	↓	↑	↓	↑	↓	↑	↓	↓	↓	↓	↑	↓
C18:0	↓			↓	↑	↓	↑	↑				
C18:1n9	↓	↓										
C15:0		↓	↓	↓						↓	↓	↓
C14:0	↓	↓	↓	↓	↓	↓						
C13:0	↑	↑										

7.2.4 IONOMICS

In the comparison of data in appendix Table 19, Copper sub-lethal exposures exhibited slight changes in most heavy metals investigated except for the two metals (Magnesium and Phosphorus), which had a more increasing affect. For Zinc it had been shown that different sub-lethal concentrations increased the amount of Silver and Phosphorus, while it decreased the remaining elements in the inorganic profile. Aluminium caused changes to the elemental profiles of whole nematodes for the two lowest concentrations. We found large significant increases in multiple metals known to cause oxidative stress (e.g.; Ag, Co, Dy, Er, Eu, Fe, Gd, Ho, La, Nd, P, Pr, Rb, Se, Sm, Sn, Tb, Th, Tl, Tm, U, W and Y), while the last two highest Aluminium doses caused decrease in the elemental profile. In general, we have found that Copper, Zinc and Aluminium have caused reduction in most elemental profiles except at the lowest Aluminium dose that caused an increased impact on the *C. elegans* elemental profiles.

7.3 DISCUSSION

7.3.1 MORTALITY

Comparing the effect of different doses of (Copper, Zinc and Aluminium) on the mortality after their respective 24 hours exposures (one-way ANOVA) revealed no significant effect of all the tested metals on the mortality of *C. elegans* ($p < 0.05$). According to the mortality test graph revealing the acute toxicity at different concentrations, the metals were also categorized into two major groups. However, the mortality percentage for Copper and Aluminium exposures were between 85% - 95%, while the mortality percentage for Zinc in the low concentrations (LC_{10} and LC_{20}) were around 95%, but in high concentrations (LC_{30} and LC_{40}) were below 80%. The discrepancy between expected and observed survival rates could once again be explained by differences in the time and temperature used for these observation (i.e. 24 hours and 20^{o}C versus 48 hours and 22^{o}C used when the original LC_{50} was determined (Section 2.4.8)). A temporal comparison of mortality has been described in Chapter 7.2.1.

7.3.2 REPRODUCTION

In general, with increasing metal concentrations, total hatched eggs were reduced. For Copper exposures, the hatched eggs in LC_{10}, LC_{20} and LC_{30} achieved 64% that was reduced and got lower in the highest dose (LC_{40}) to produce 45 hatched eggs per nematode. Hatched eggs for all Zinc concentrations were slightly stable between 53 – 56 hatched eggs. While in Aluminium exposure, the hatched egg numbers were the lowest in all exposed metals. The egg number in LC_{10} was ~ 40 hatched eggs and decreased to 34 egg in the remaining Aluminium doses.

7.3.3 METABOLOMICS

7.3.3.1 AMINO ACIDS

It has been shown in the collective results that the two highest sub-lethal Copper caused an elevation in Cystathionine and Cysteine levels, while that effect was not reported in the remaining two lowest doses. Also, it was noticed that for most of Copper concentrations Glycine and Tryptophan had been reduced, while BAIBA and Histidine were increased. For sub-lethal Zinc exposures in *C.elegans* have been generally increased most of the metabolite (amino acids) compositions increased except for BAIBA, Leucine, Isoleucine and Glutamic acid, which decreased. In addition, Aluminium had an impact on some metabolites (amino acids) by dropping their amounts in *C. elegans,* with the exception of Aspartic acid, Hydroxylysine and Glutamic acid, which increased. Furthermore, the highest dose caused an increase in all of the Amino acids detected.

7.3.3.2 FATTY ACIDS

Our findings, demonstrated that Copper had decreased the total fatty acids content in *C. elegans* except, for one fatty acid, which was C18:2n6. Zinc had a remarkable effect on the nematode fatty acids profile, whereby most were reduced in concentration except for C18:0 that increased in concentration (versus controls). Table 16 demonstrated that Aluminium caused decrease in most fatty acids except for C14:0 and C20:4n3 at the lowest Aluminium sub-lethal exposure dose. In general, it has been observed from the comparison that Copper, Zinc and Aluminium had a significant impact on fatty acids by decreasing their concentrations in *C. elegans*.

7.3.4 IONOMICS

In the comparison, Copper sub-lethal exposures exhibited slight changes in most heavy metals investigated except for the two metals (Magnesium and Phosphorus), which had a more increasing affect. For Zinc it had been shown that different sub-lethal concentrations increased the amount of Silver and Phosphorus, while it decreased the remaining elements in the inorganic profile. Aluminium caused changes to the elemental profiles of whole nematodes for the two lowest concentrations. We found large significant increases in multiple metals known to cause oxidative stress (e.g.; Ag, Co, Dy, Er, Eu, Fe, Gd, Ho, La, Nd, P, Pr, Rb, Se, Sm, Sn, Tb, Th, Tl, Tm, U, W and Y), while the last two highest Aluminium doses caused decrease in the elemental profile. In general, we have found that Copper, Zinc and Aluminium have caused reduction in most elemental profiles except at the lowest Aluminium dose that caused an increased impact on the *C. elegans* elemental profiles.

8 MIXTURE EXPOSURES TOXICITY ASSESSMENT IN *CAENORHABDITIS ELEGANS* AFTER SUB-LETHAL EXPOSURES

8.1 INTRODUCTION

The metals that intoxicate the environment are usually present in the form of metal mixtures in the environment, majorly containing toxic metal cations. According to Toro & Allen (2001), at the level of biotic ligands H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} compete with toxic ions, such as Cu^{2+} and Zn^{2+}, for binding sites. This competition is healthy as it results in the reduction of accumulation of toxicants, and hence, in metal toxicity. Cations are known to be directly associated with metal toxicity. Little research has been done in the regard which effectively justifies the contribution of the ion-ion interactions in predicting bioaccumulation and toxicity of mixtures of toxic metals (Borgmann, 2008). When large amounts of cations are present, they decrease the negativity of the electric potential at the membrane surface, which not only effects the activity of toxic ions at the PM surface but also hinder effective transportation of various ions through the membrane.

According to Kabata-Pendias (2000), the interaction between the metals in the mixtures may occur at the environment level, at the root surface or within the plant. When these interactions take place at environmental level, it can be seen that the magnitude of interactions depend upon the amount of metal ions present in the environment. The physicochemical conditions of the environment also plays an important role in initiating possible interactions. But even when the interaction occurs between the metal components which are present in their lowest quantities, still they are capable enough to result in serious adverse effects (Cooper *et al.*, 2009). The harmful effects of these

interactions show themselves both toxic kinetically and dynamically by affecting the substance uptake due to interactions among different substances and by affecting the joint toxicity due to target site interactions in organisms, respectively.

8.2 RESULTS

8.2.1 MORTALITY

To derive the lethality profile of our studied metals mixtures within out test system, we performed 24 hours exposure of adult nematodes (See Section 2.4.8). Comparing the effect of different metal mixtures on the mortality of *C. elegans* between subjects, a one-way ANOVA was conducted to compare the effect of different doses of different mixtures in this study on the mortality of *C. elegans* Figure 40. There was no significant effect of all different metals doses on the mortality of *C. elegans* ($p < 0.05$ level).

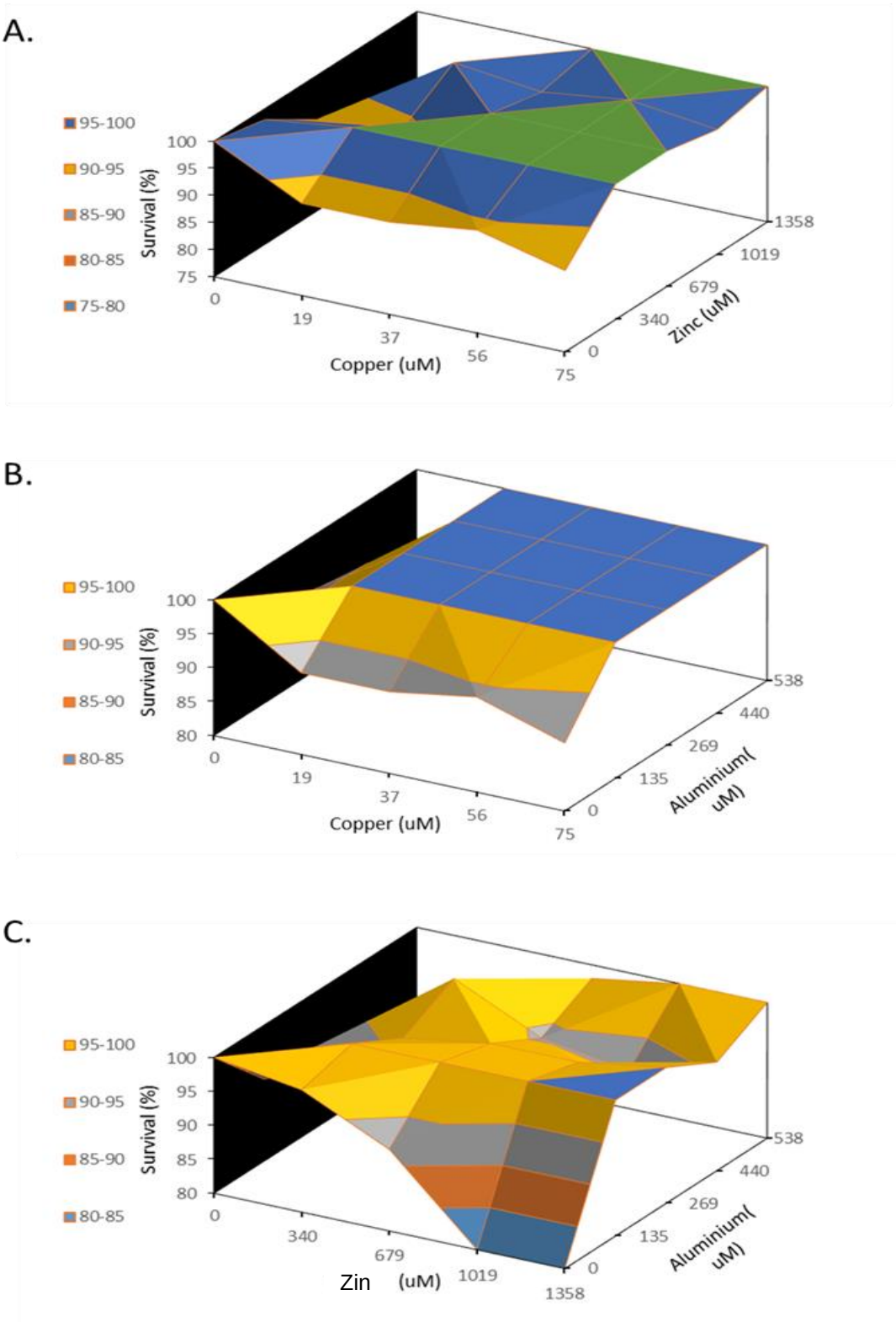

Figure 40: Survival impact of metal mixtures on *C. elegans* after 24 hours exposure. 10 adults nematodes from age-synchronized cultures exposed to various concentrations of Copper & Zinc (Panel A), Copper and Aluminium (Panel B) and Zinc and Aluminium (Panel C). The number of survivals was counted. For each test concentration and control, the average number of survivals from the wells was obtained for each trial, and at least five trials were conducted after their **exposure** with different doses of Copper in 24 hours period.

8.2.2 REPRODUCTION

To evaluate the sub-lethal impact of Aluminium on reproduction we performed a 72 hours exposure using age synchronized cultures (See Section 2.4.9) and employing the identical exposures levels based on previous literature and described in Section 4.2.1 above. In metal mixtures Figure 41, it was noticed that all mixtures had a significant reduction impact on the numbers of *C. elegans's* eggs laid after the exposure test. Noticeably, the most effect was found with the Copper/Aluminium and Zinc/Aluminium exposures tests, while Copper/Zinc mixtures had the lowest impact.

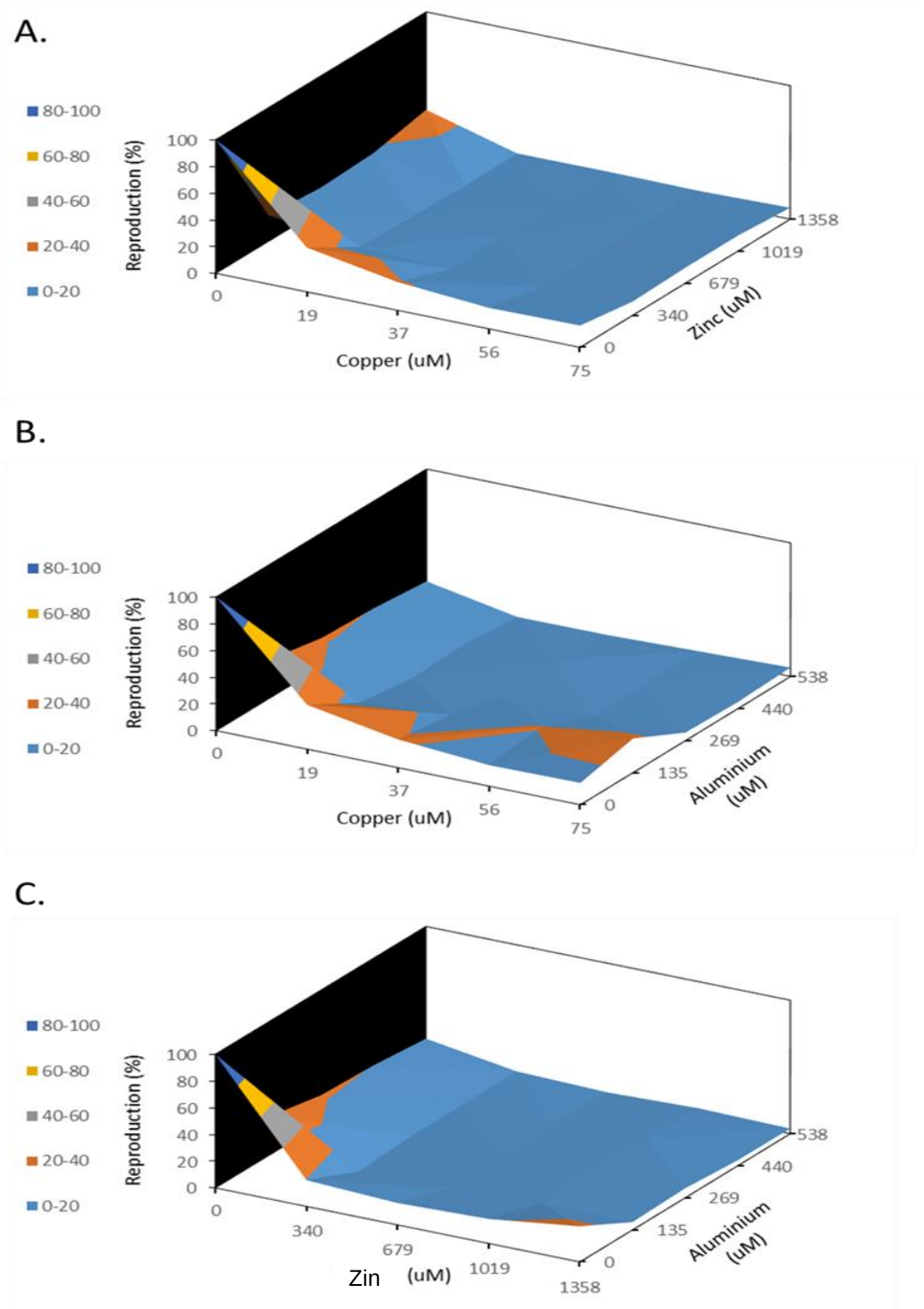

Figure 41: Reproduction impact of metal mixtures on *C. elegans* after 72 hours exposure. Single nematode from age-synchronized cultures exposed to various concentrations of Copper & Zinc (Panel A), Copper and Aluminium (Panel B) and Zinc and Aluminium (Panel C). The number of offspring at all stages beyond the eggs was counted. For each test concentration and control, the average number of progenies from the wells was obtained for each trial, and at least five trials were conducted.

8.2.3 METABOLOMICS

8.2.3.1 AMINO ACIDS

C. elegans nematodes were exposed to designed metals mixtures and the metabolites in the aqueous phase, i.e. Amino Acids and ions in the organic phase were all determined via GC-MS technique. For the Copper/Zinc mixture test Figure 42, the interaction between the Copper and Zinc has an impact on the amino acids profiles, determined elevated concentration versus the control sample. Noticeably, Cysteine was the most increased amino acid in all our designed treatments, whereas Glycine and Glutamic acid were the most decreased amino acids. Copper/Aluminium mixture test resolved that the interactions between three lowest Aluminium doses (LC_{10}, LC_{20} and LC_{30}) with all Copper doses decreased most amino acids in the nematode Figure 43. In contrast, highest Aluminium dose (LC_{40}) mixed with all Copper doses were elevated the amount of nearly all amino acids in *C. elegans*, except Lysine, which was decreased. No exact trend was detected Figure 44 for the Zinc/Aluminium mixture test. Although, most amino acids decreased.

8.2.3.1.1 Cu/Zn exposures

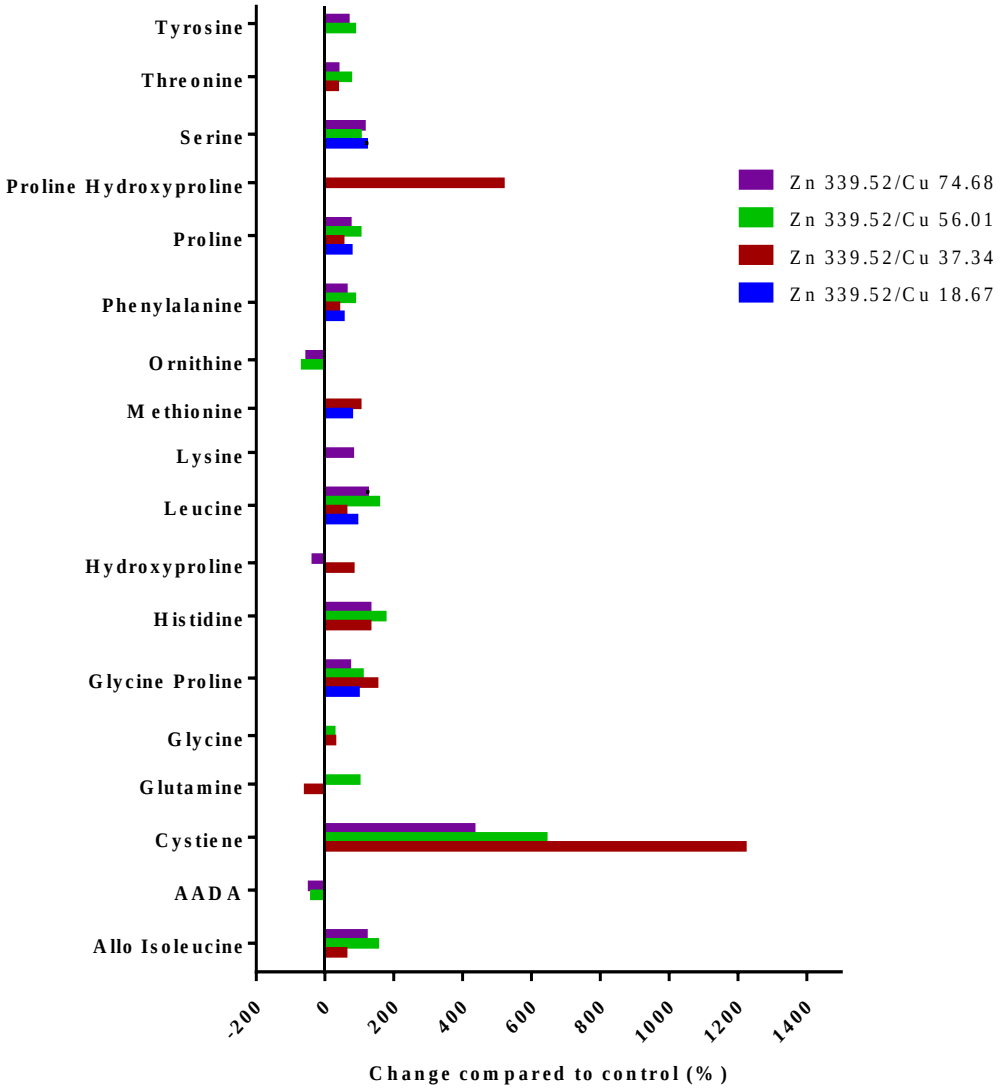

Figure 42: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{10}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

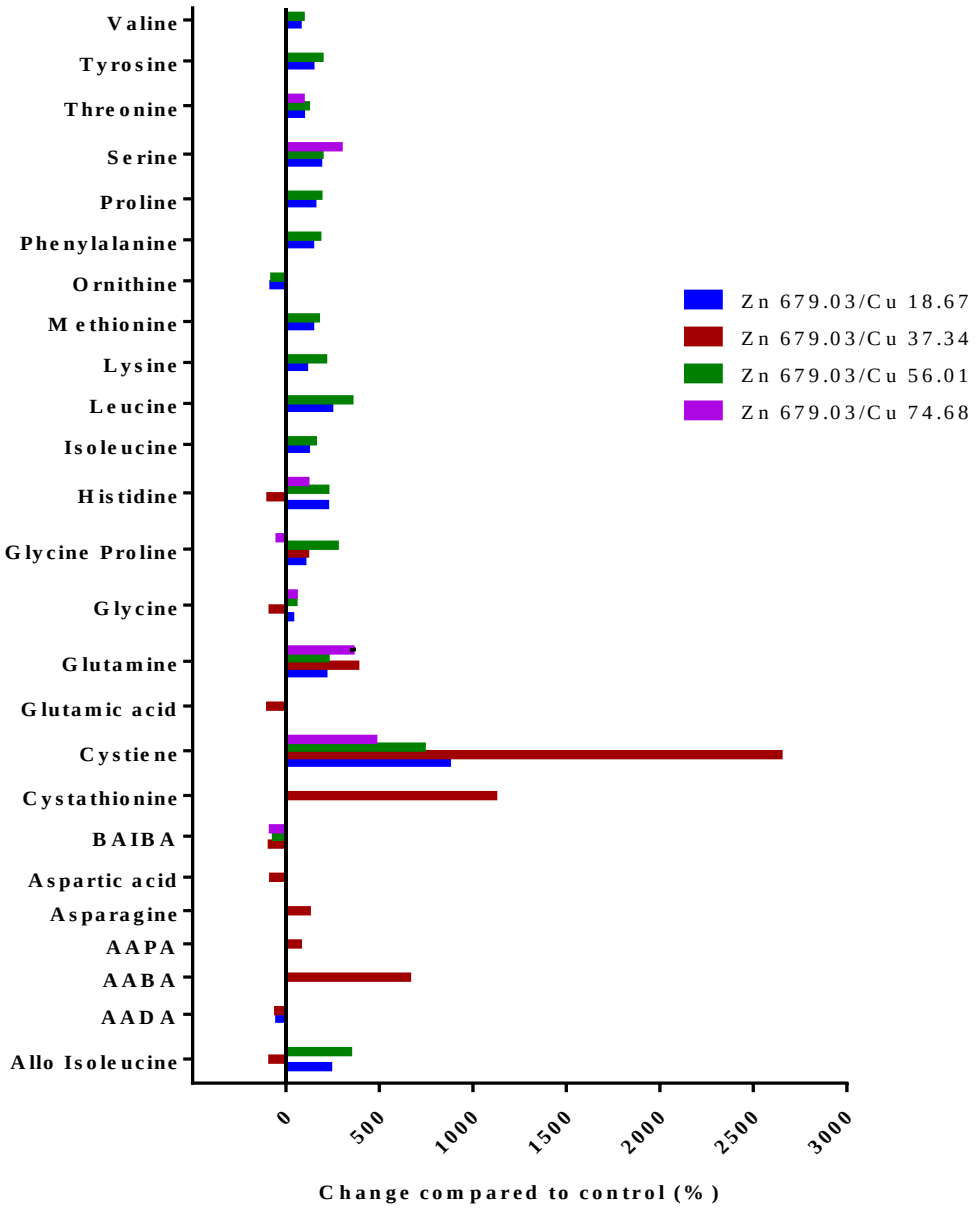

Figure 42 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{20}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

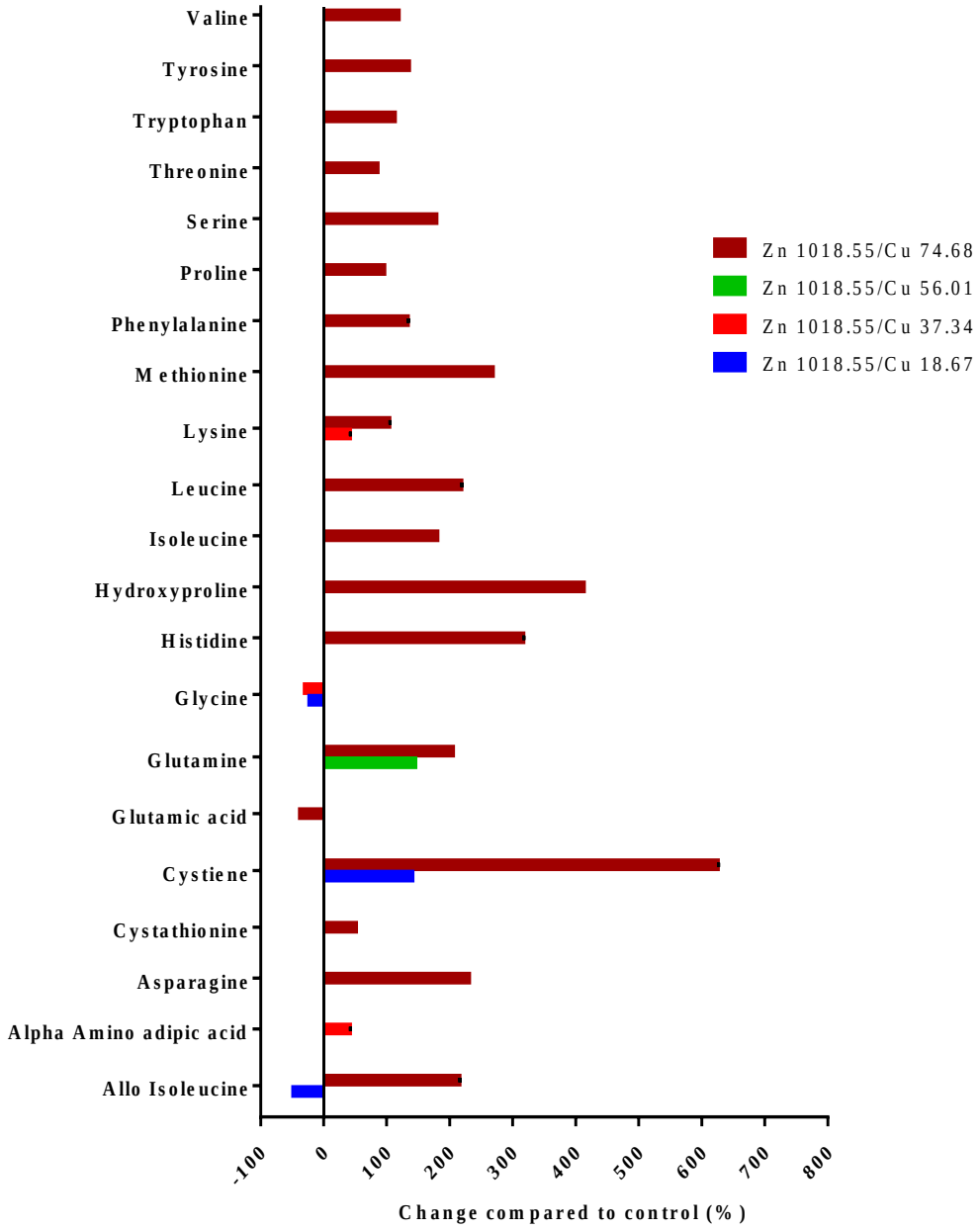

Figure 42 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{30}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

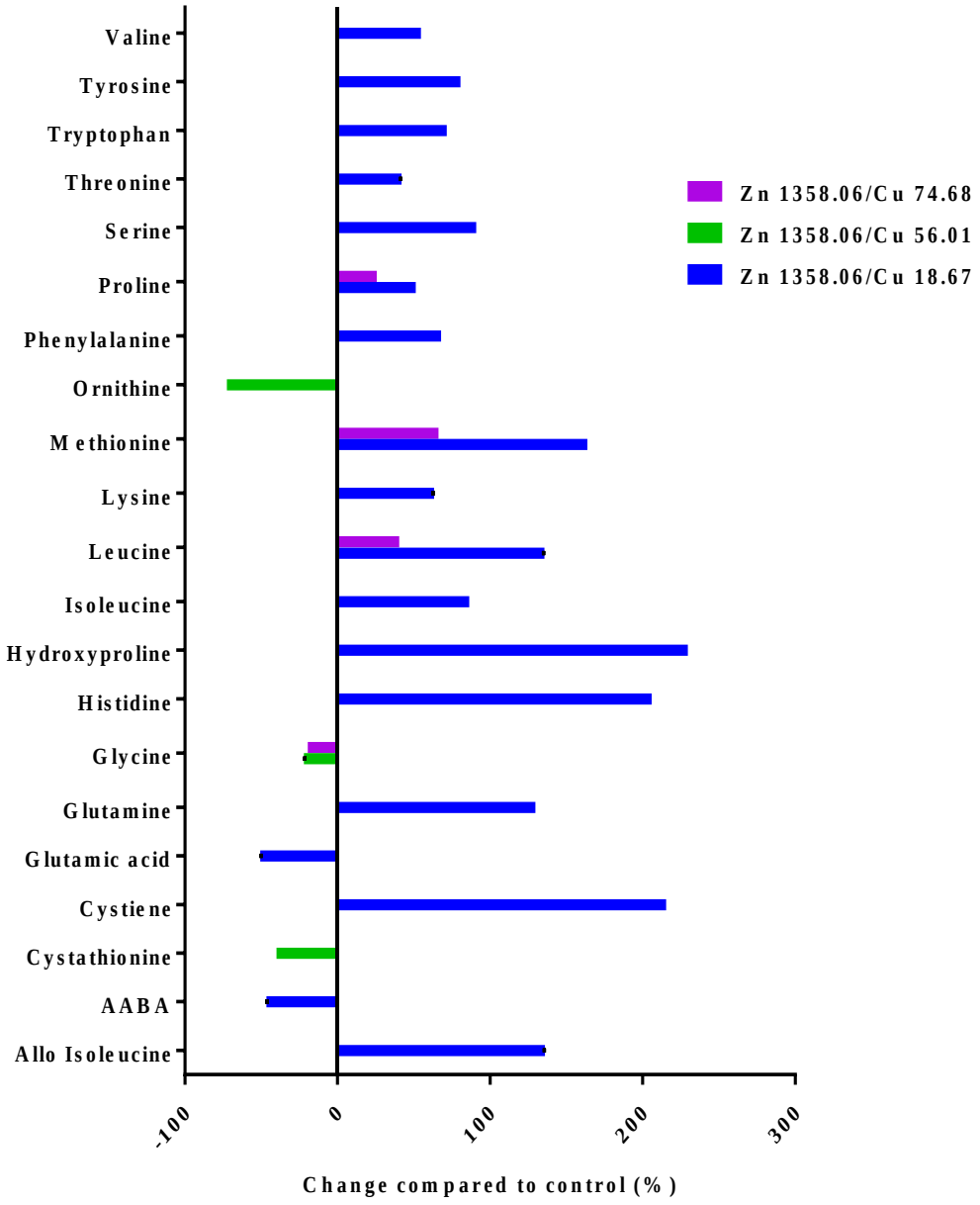

Figure 42 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{40}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

8.2.3.1.2 Cu/Al exposures

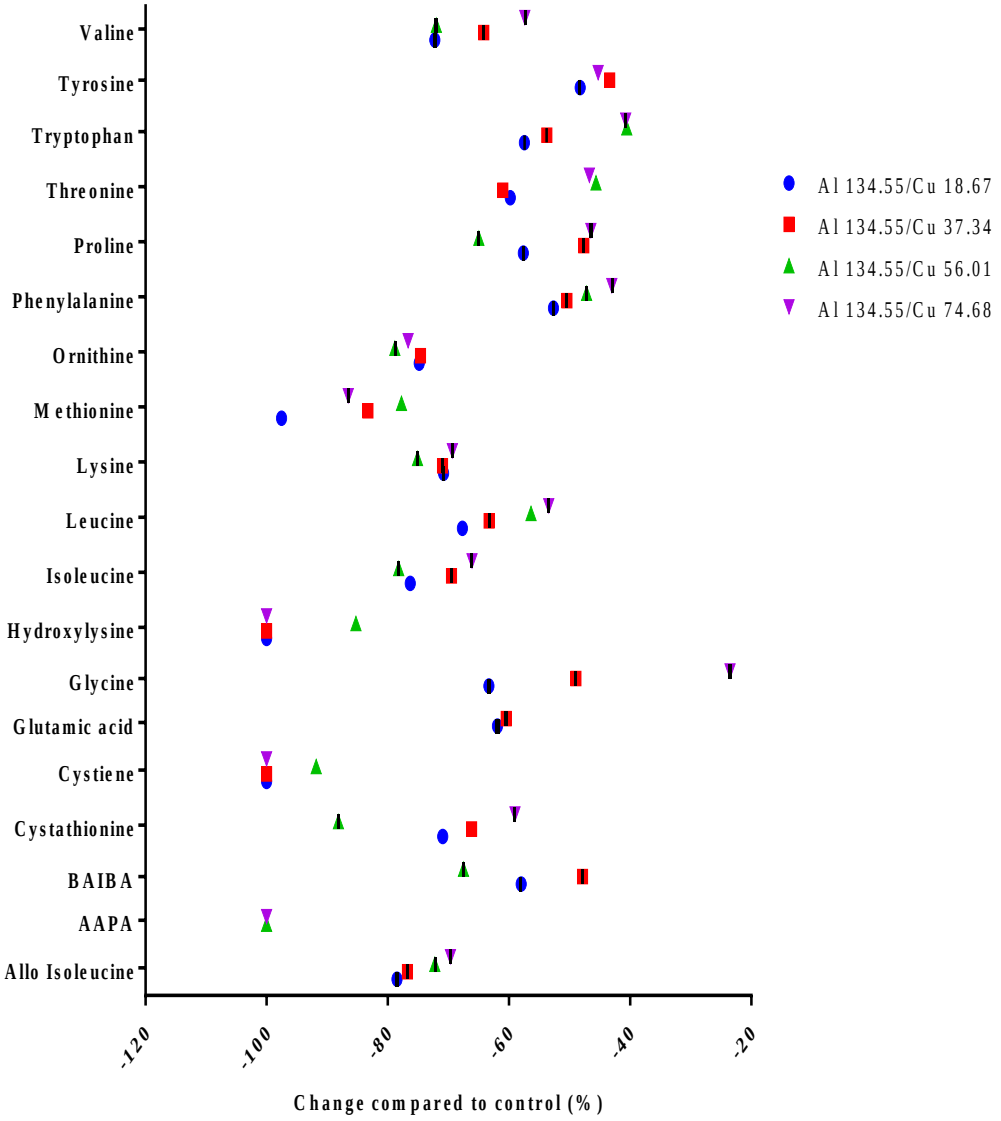

Figure 43: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{10}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

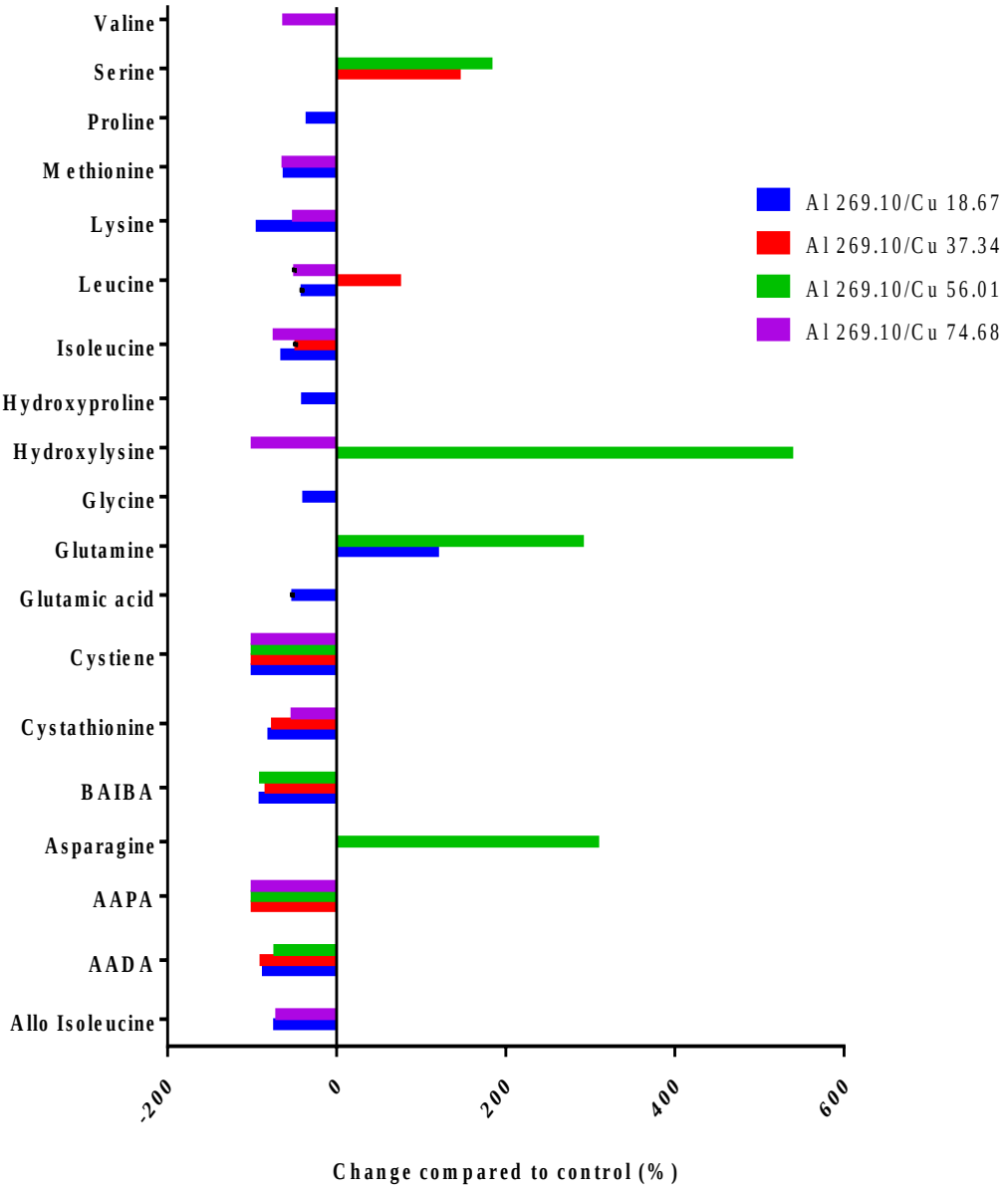

Figure 43 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{20}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

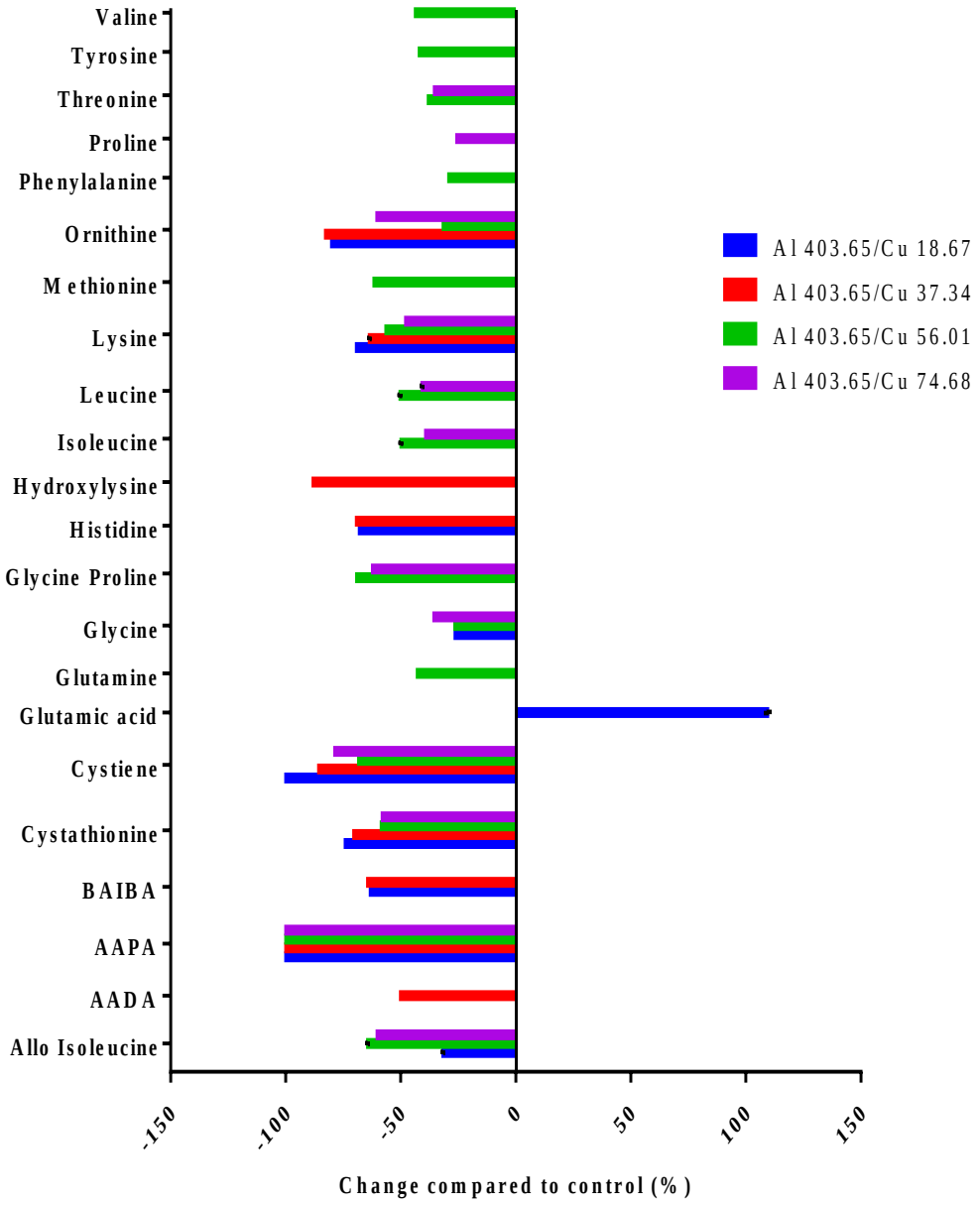

Figure 43 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{30}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

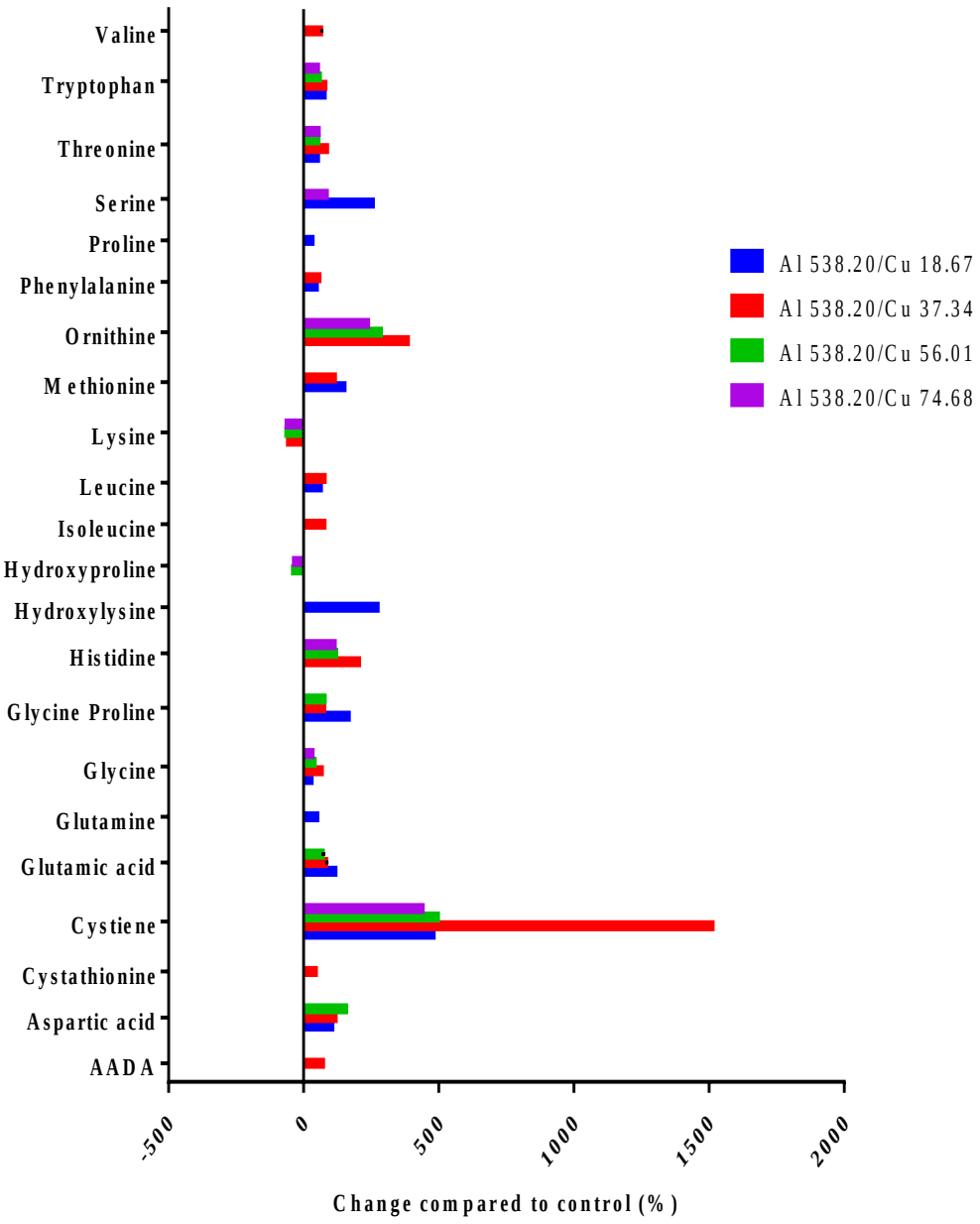

Figure 43 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{40}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

8.2.3.1.3 Zn/Al exposures

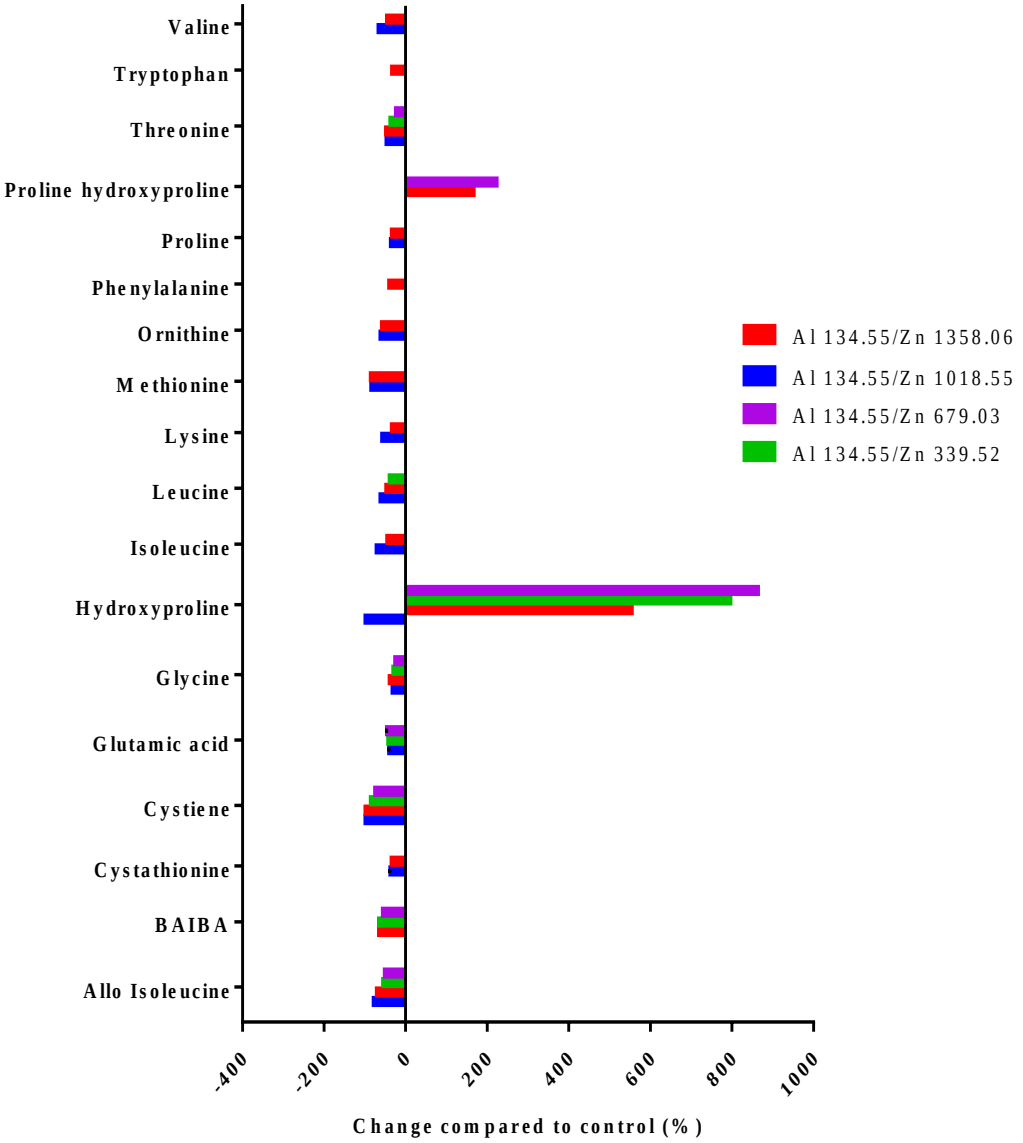

Figure 44: Changes in amino acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{10}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

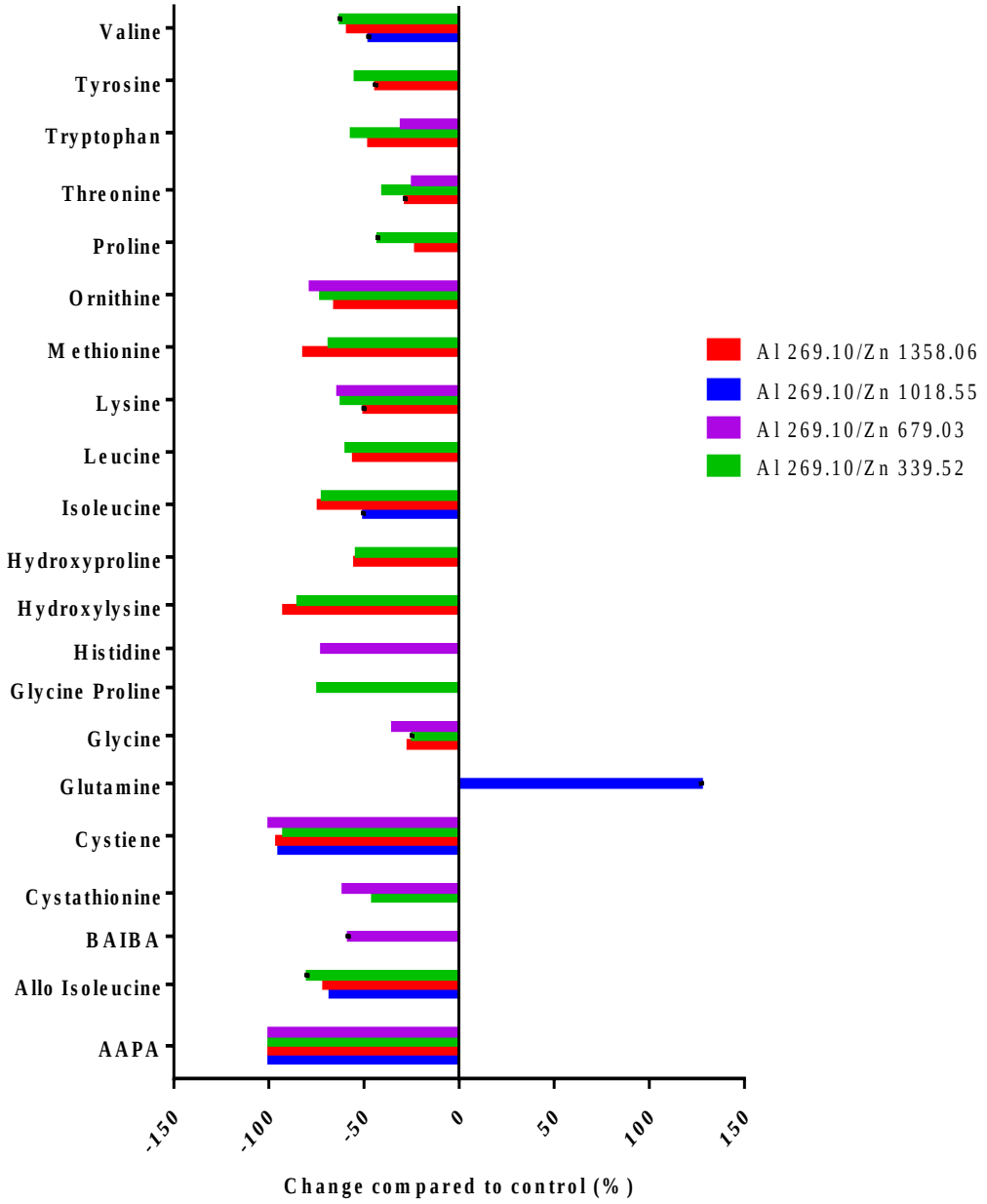

Figure 44 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{20}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

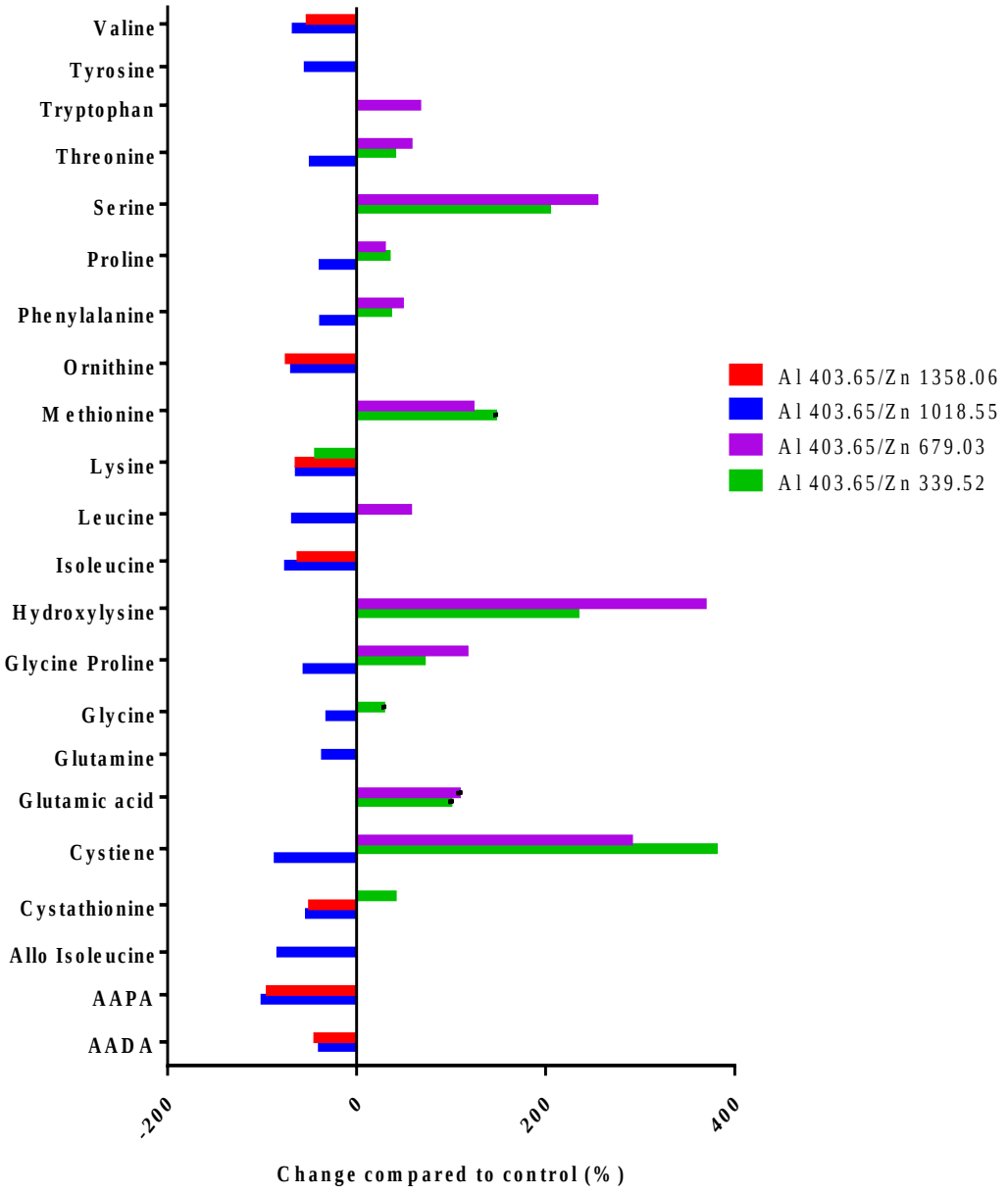

Figure 44 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{30}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

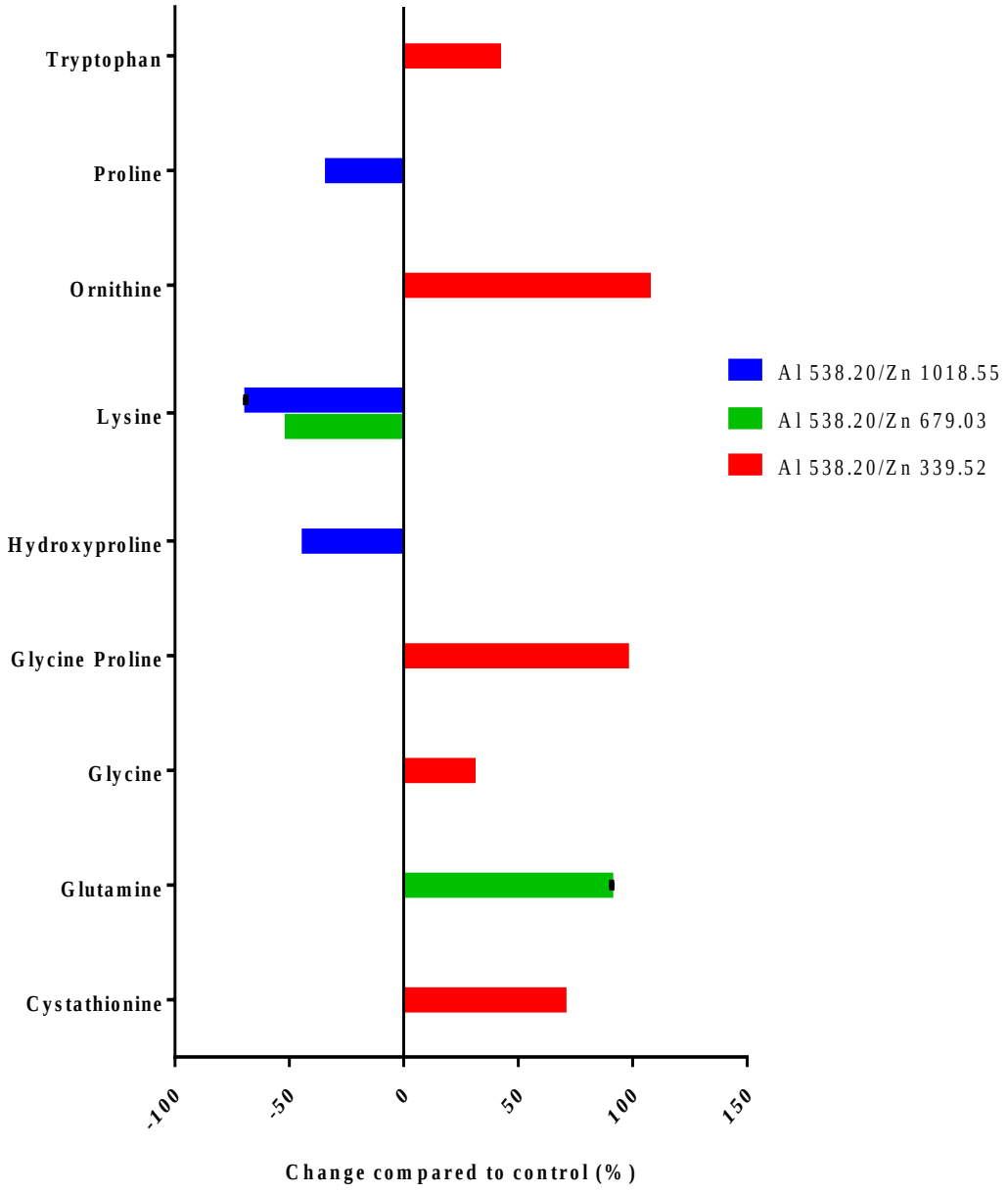

Figure 44 Continued: Changes in amino acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{40}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

8.2.3.2 FATTY ACIDS

C. elegans nematodes were exposed to designed metals mixtures and the metabolites in the aqueous phase (Amino Acids) and metabolites in the organic phase were all determined via GC-MS technique.

In Figure 45, Copper/Zinc mixture was noticeably decreased with nearly all fatty acids in all our designed test, except some fatty acids, increased in some mixture treatments such as C20:0 in Zinc (LC_{30}) mixed with Copper (LC_{40}) designed treatment and C20:4n3 in Zinc (LC_{20}) mixed with Copper (LC_{20}). The same trend has been shown in Copper/Aluminium and Zinc/Aluminium mixture tests in Figure 46 and 47 respectively, where nearly all fatty acids were decreased except C15:0, which rose in two designed treatments for the Aluminium doses (LC_{30} and LC_{40}) mixed with all Copper and Zinc sub-lethal doses. However, the Zinc/Aluminium mixture Figure 47 determined fatty acids were slightly increased (e.g. C14:0, C17:0, C18:0 and C20:0).

8.2.3.2.1 Cu/Zn exposures

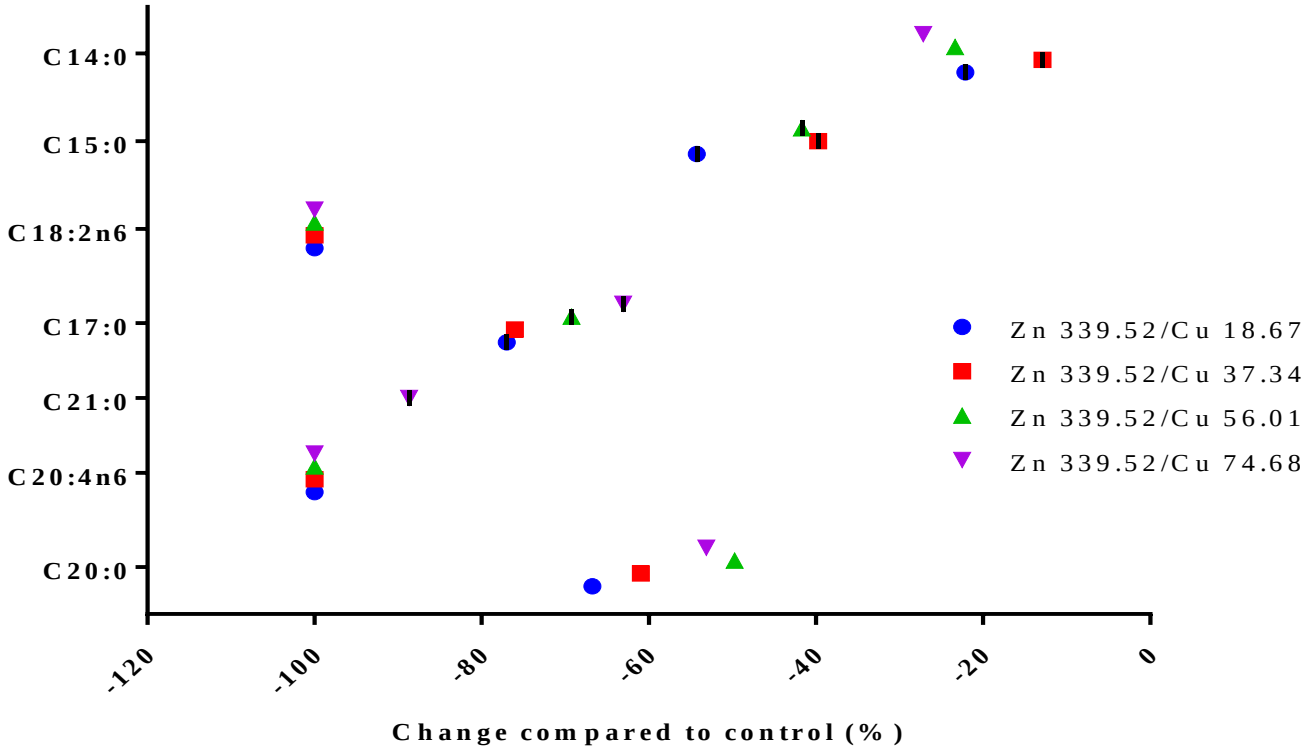

Figure 45: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{10}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

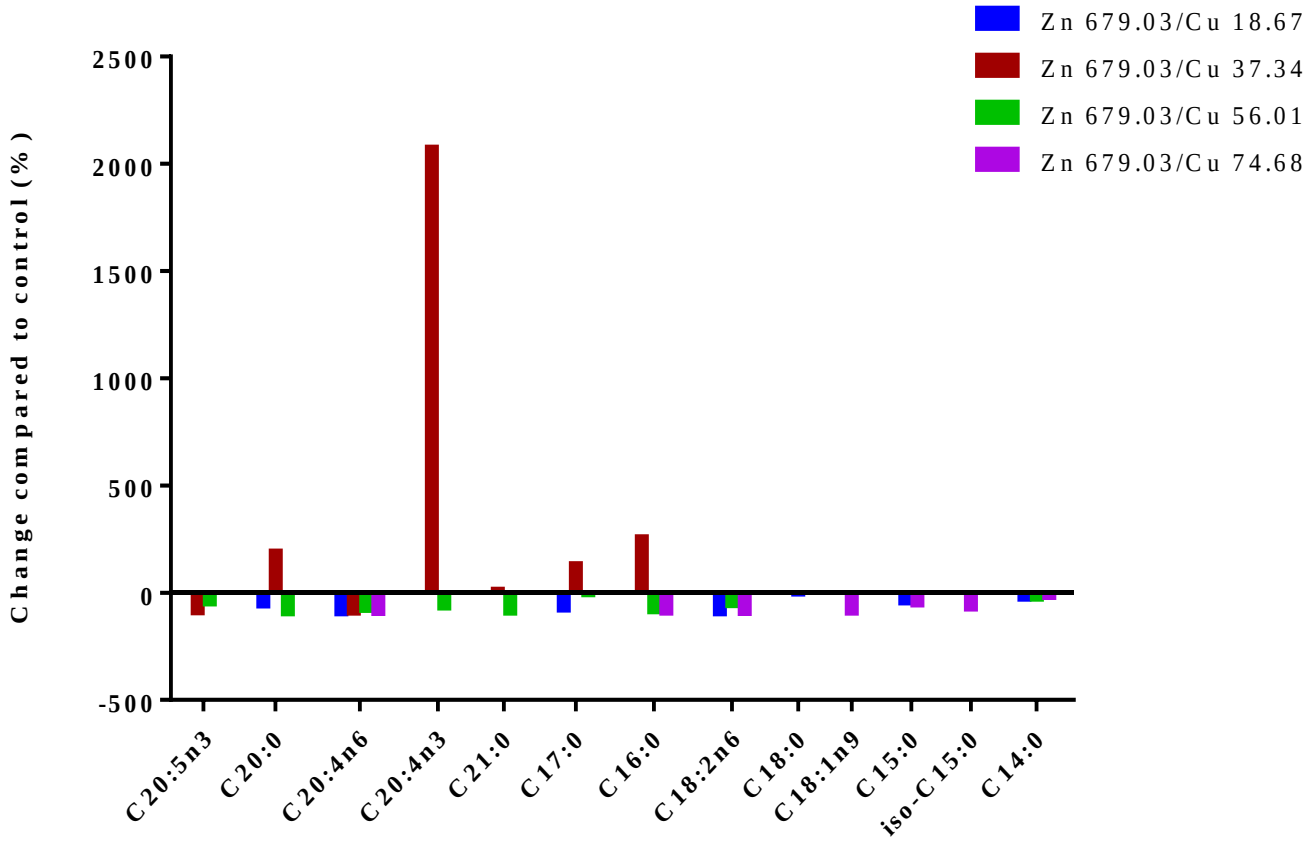

Figure 44 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{20}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

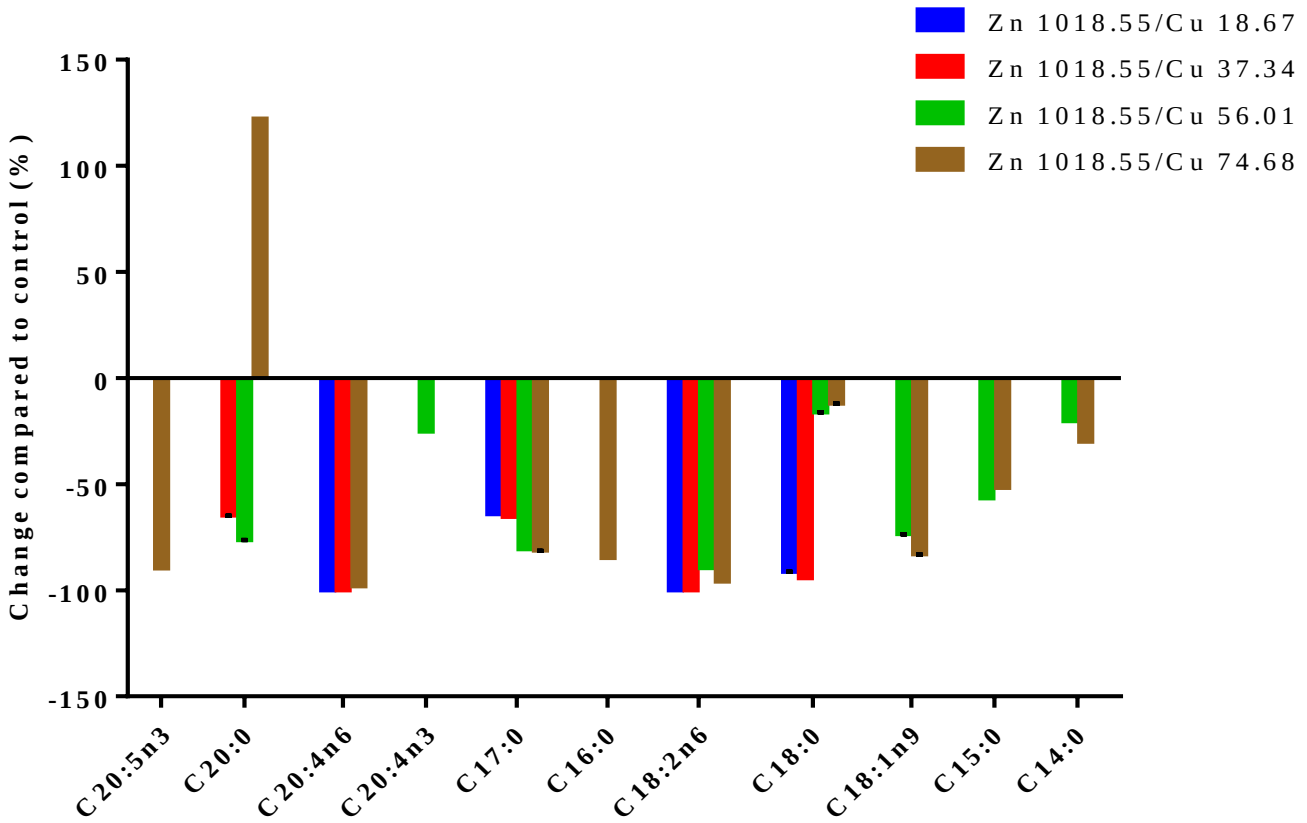

Figure 44 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{30}) exposed and compared to control sample. All bars in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

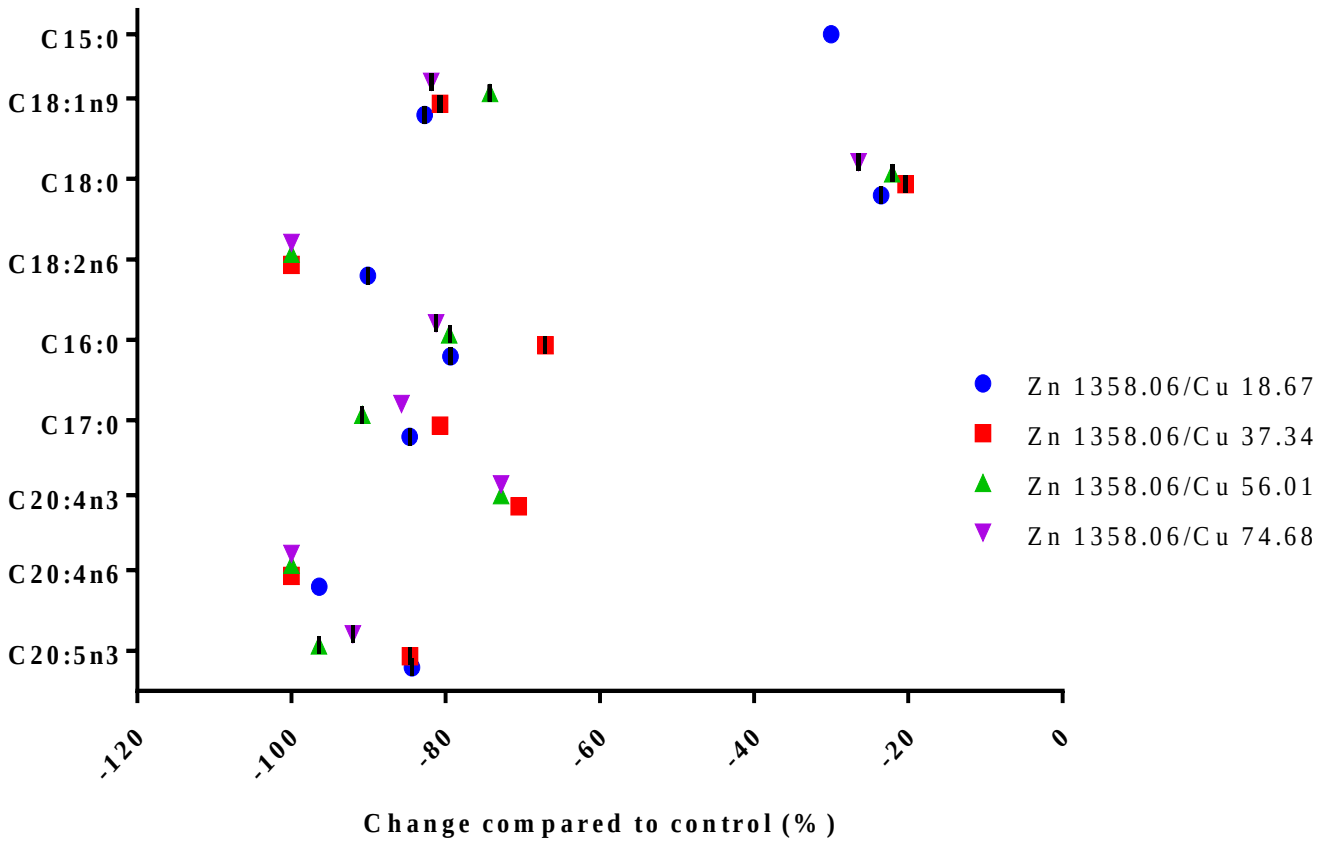

Figure 44 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Zinc dose (LC_{40}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

8.2.3.2.2 Cu/Al exposures

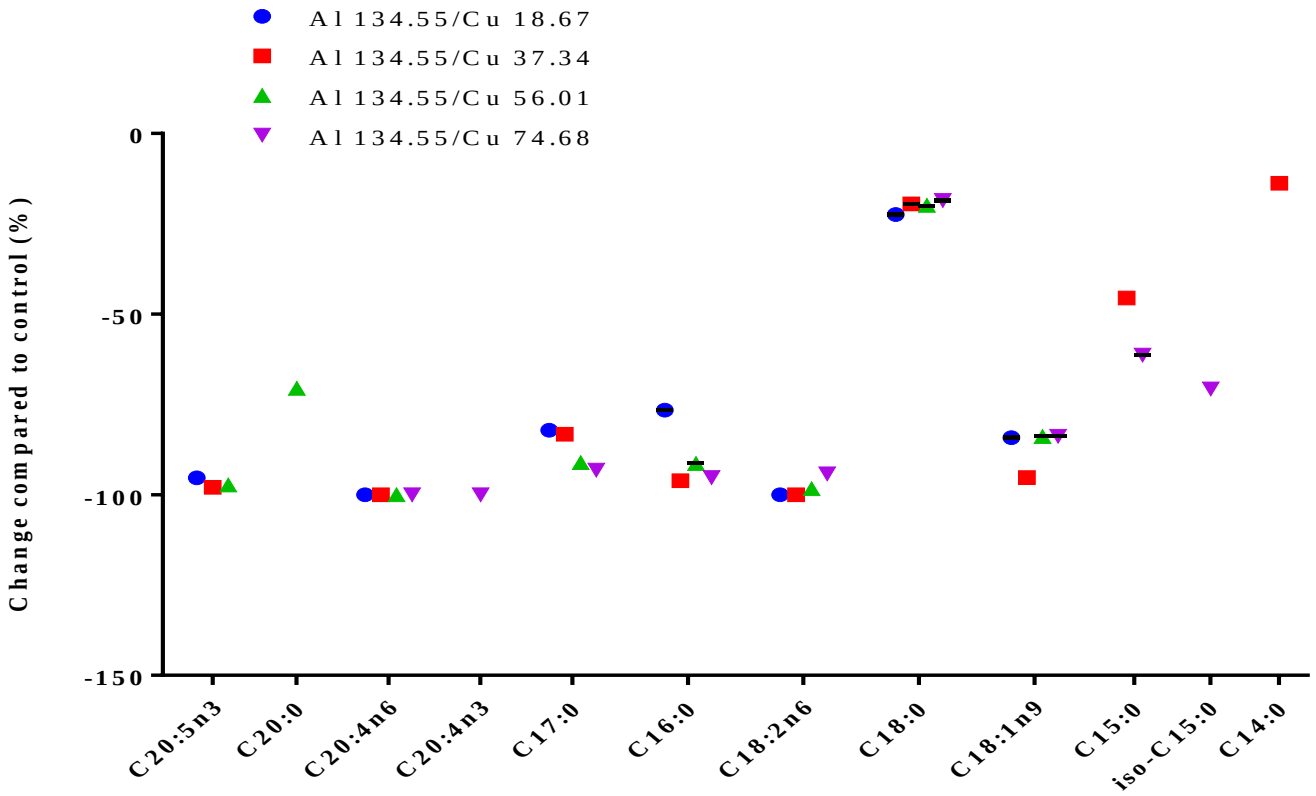

Figure 46: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{10}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

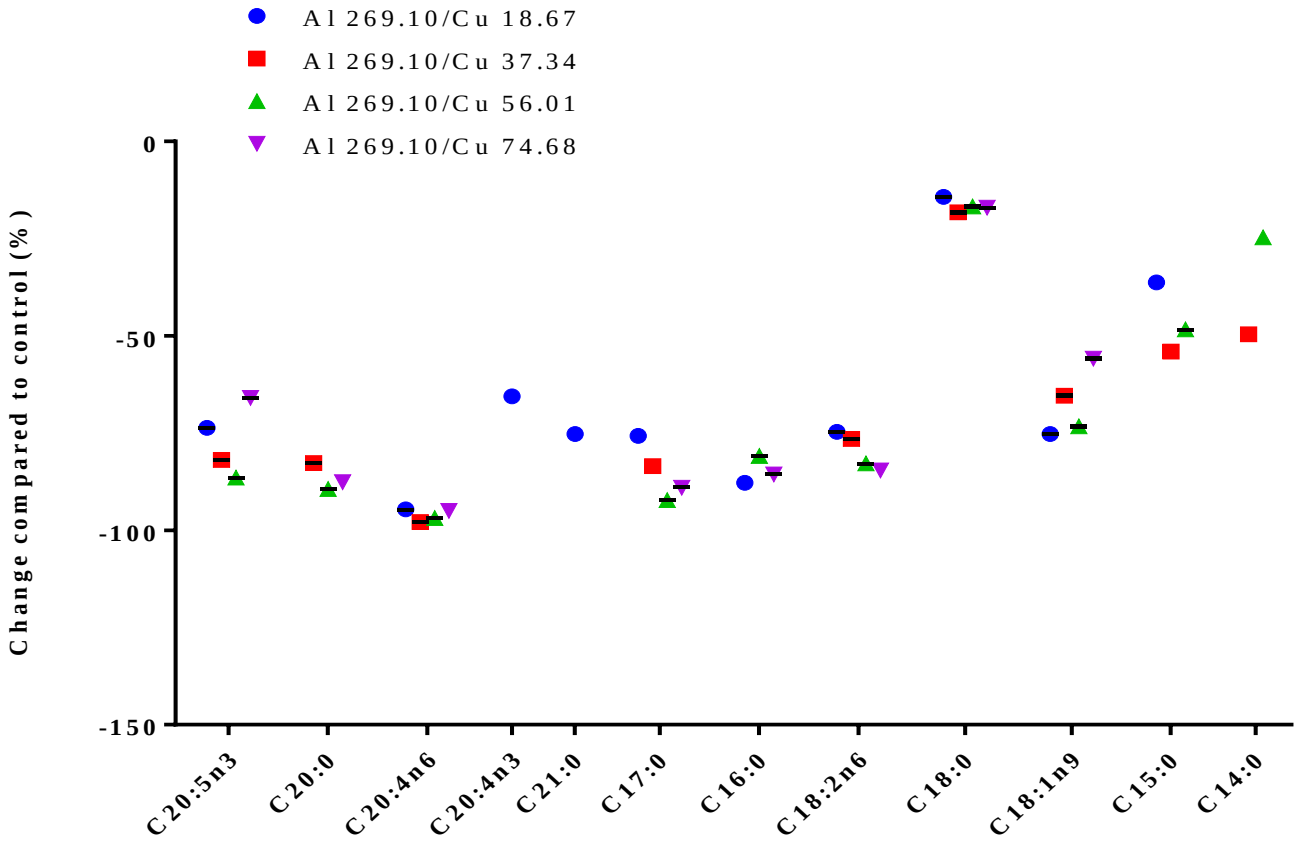

Figure 46 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{20}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

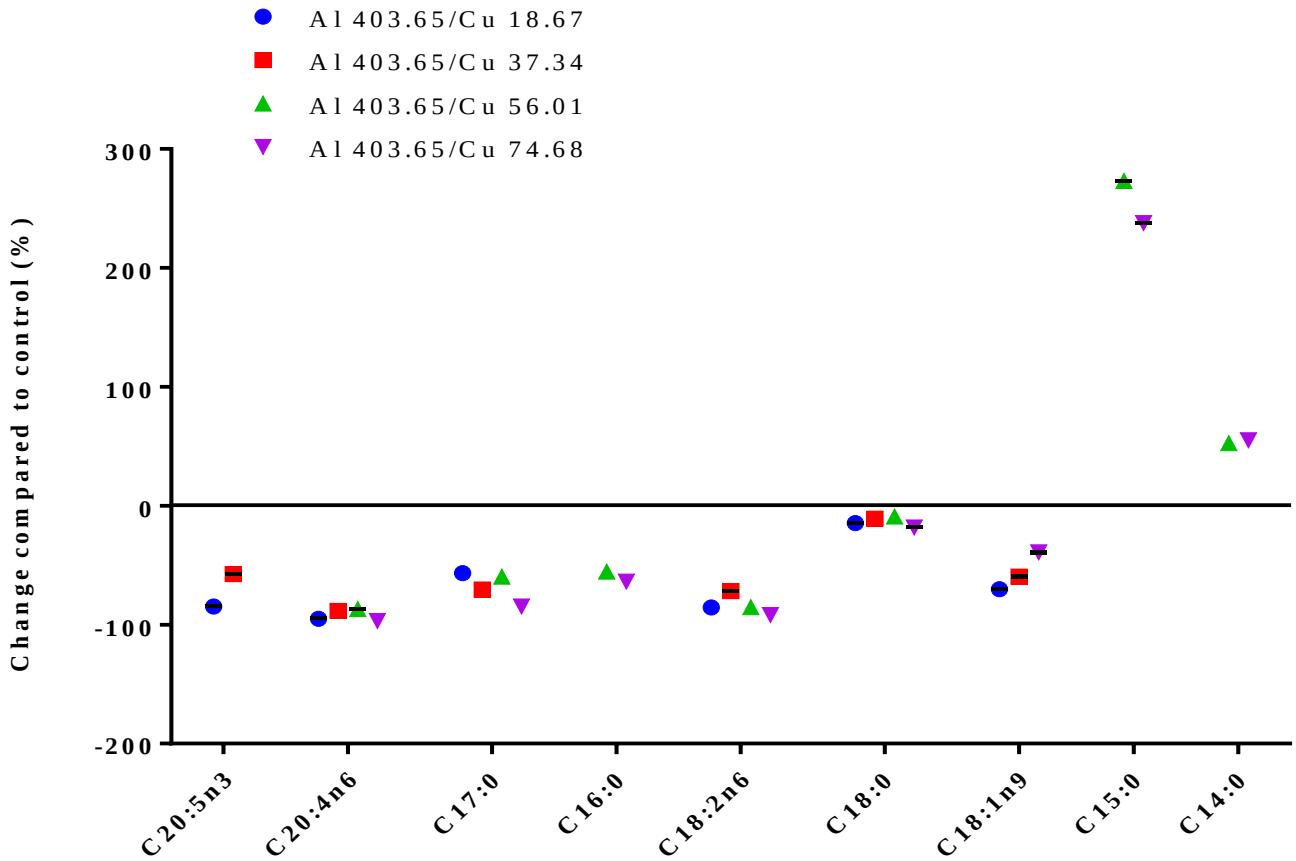

Figure 46 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{30}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

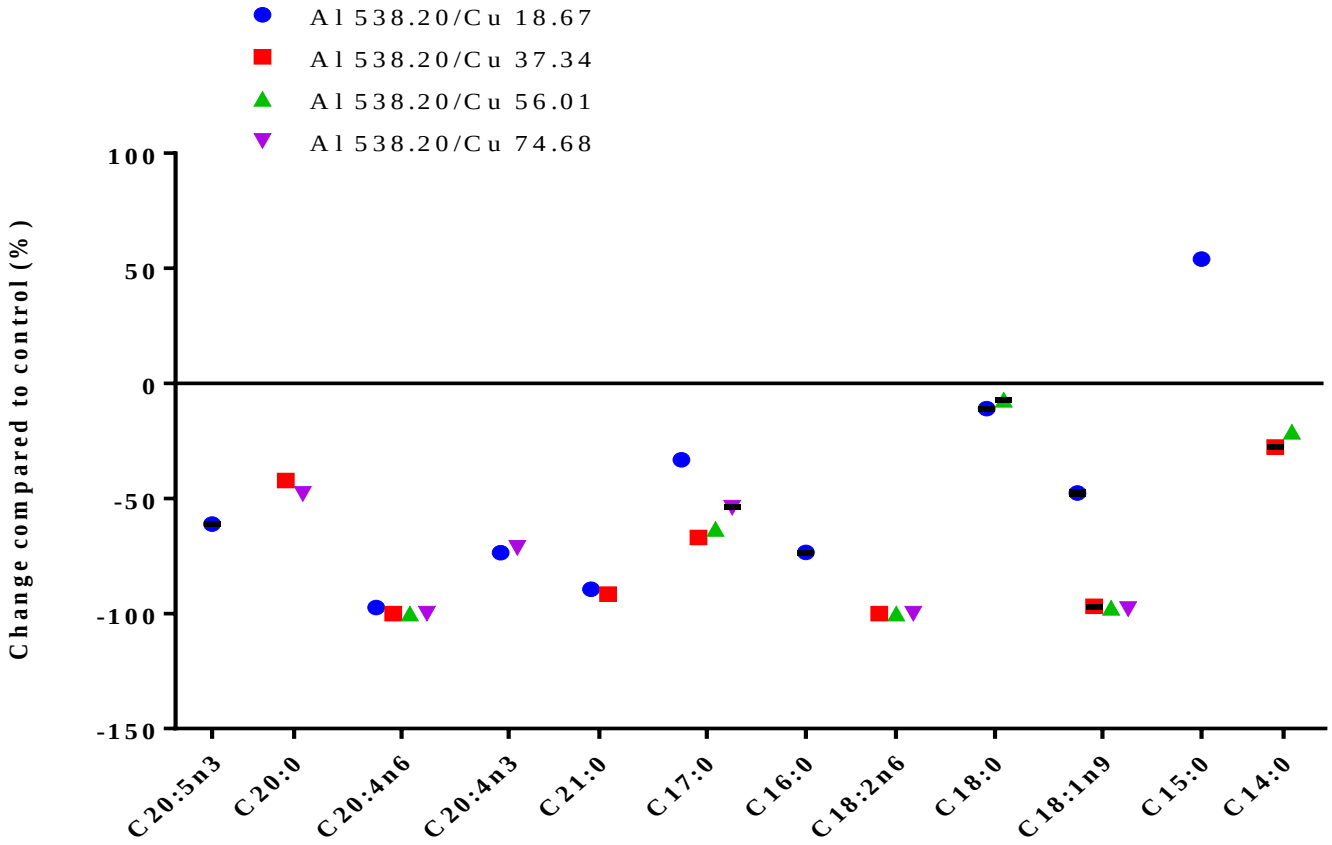

Figure 46 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Copper different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{40}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

8.2.3.2.3 Zn/Al exposures

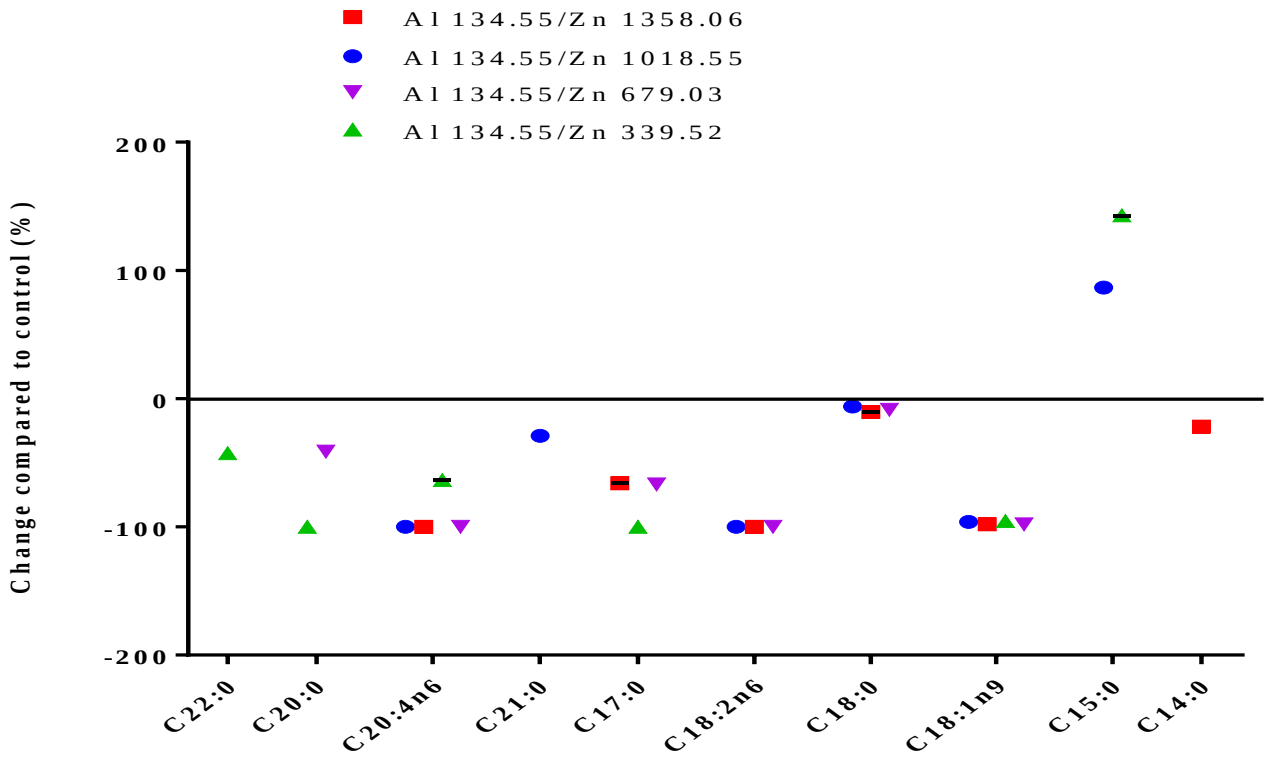

Figure 47: Changes in fatty acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{10}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

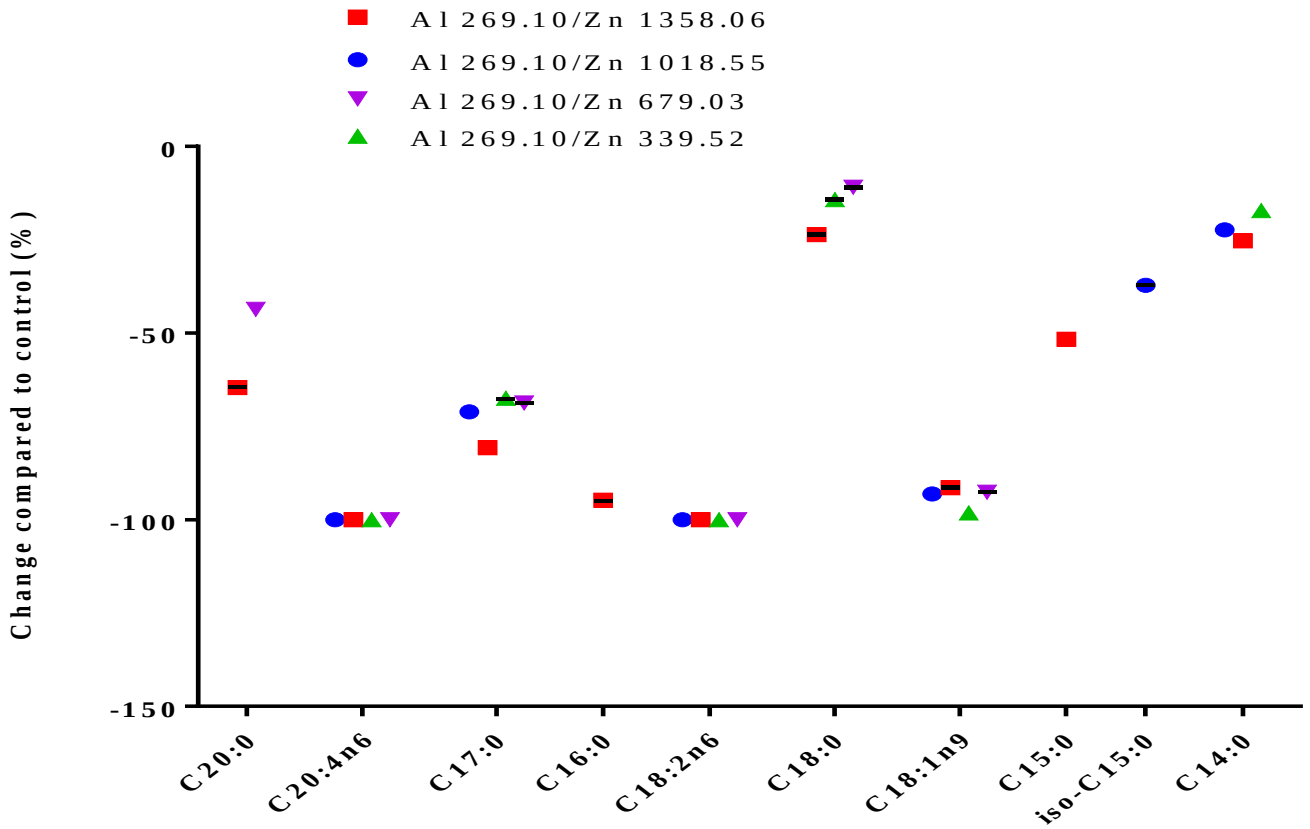

Figure 47 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{20}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

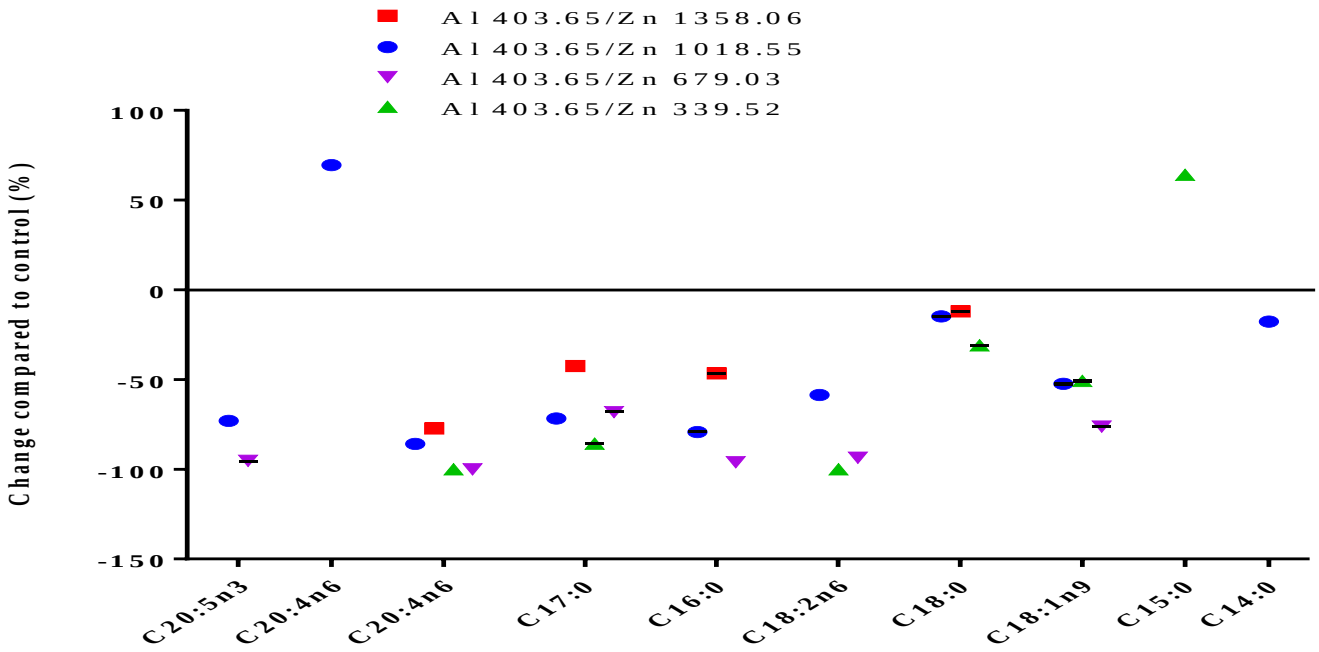

Figure 47 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{30}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

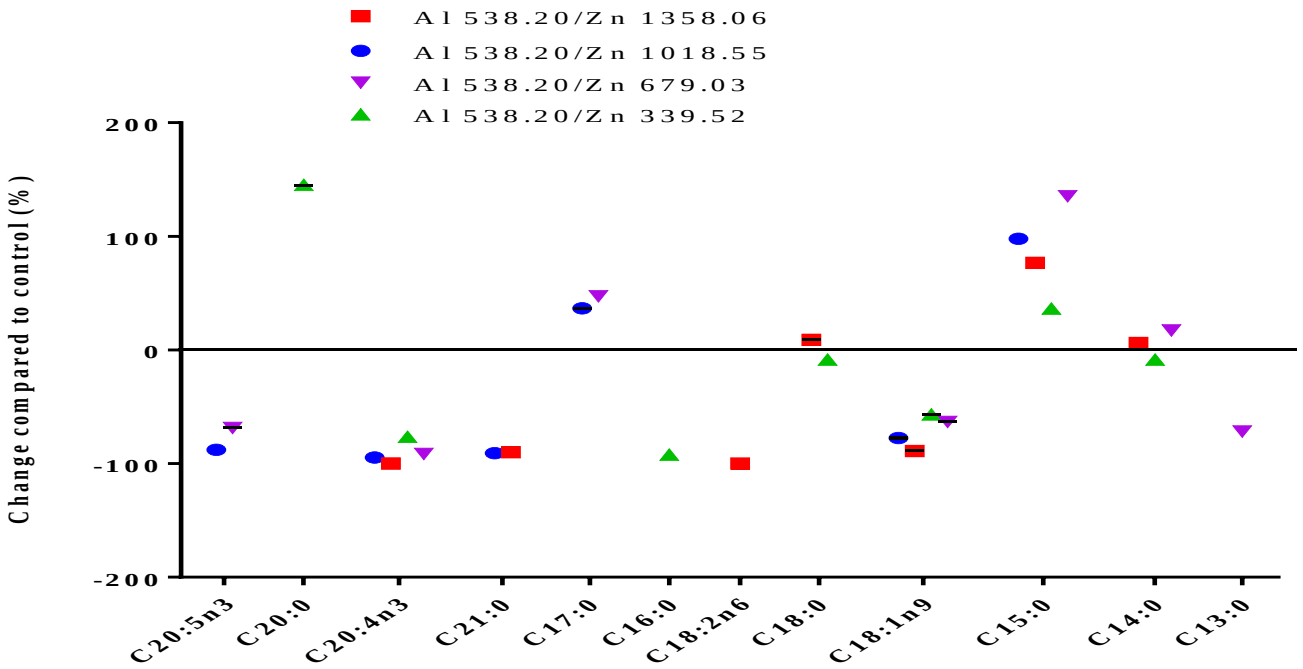

Figure 47 Continued: Changes in fatty acids composition measured by GC-MS after 24 hours Zinc different doses (LC_{10}, LC_{20}, LC_{30} and LC_{40}) with Aluminium dose (LC_{40}) exposed and compared to control sample. All points in the chart represents significant difference percentage when compared to control levels ($p < 0.05$).

8.2.4 IONOMICS

Investigating the elemental profile changing in *C. elegans* after metals mixture exposures were analysed via the ICP-MS, Figure 48, the data showed the impact of Copper/Zinc mixtures. Generally, all the elements decreased in all mixture designs except the Silver that increased in all designed exposures. The same trend was shown for Copper/Aluminium mixtures in Figure 49 where all elements decreased except Phosphorus and Zinc, which increased instead of Silver in the Copper/Zinc mixture. For Zinc/Aluminium mixtures Figure 50 demonstrated a drop in most of the element profiles except for Copper and Phosphorus, which increased in most of the designed exposures. In addition, Gallium was noticeably slightly increased in most of the Zinc/Aluminium mixtures.

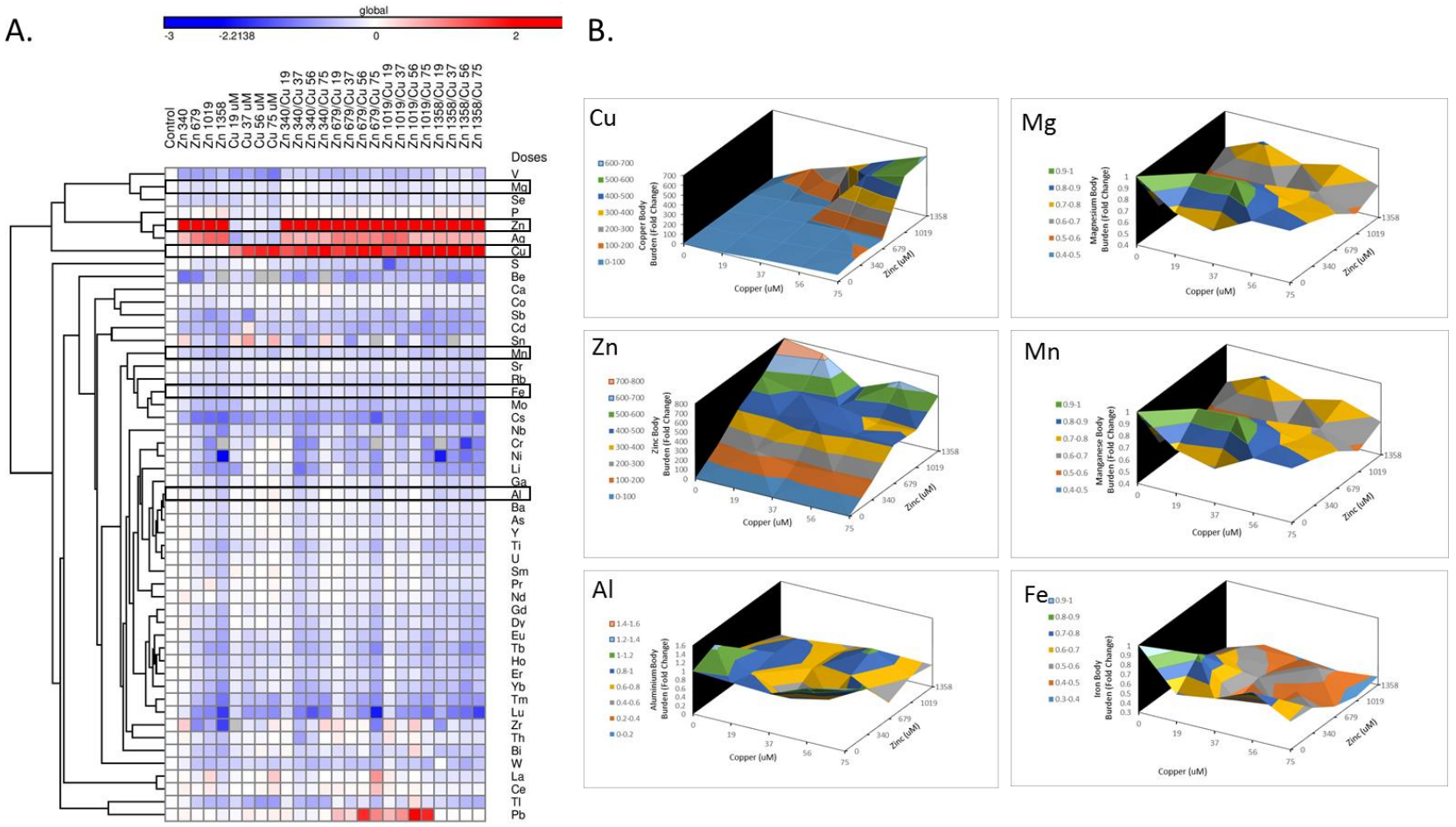

Figure 48: Changes in elemental composition of nematodes exposed to mixture of Copper and Zinc. Nematodes were from age-synchronized cultures exposed to various concentrations of Copper and Zinc. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 h period. Panel A: Hierarchical clustering of changes in ions levels in response to Cu/Zn mixtures. Log2 (Mean fold change) is clustered for ion using a minus one Pearson correlation with average linage method applied. Panel B: Example topographic profiles for the impact of mixtures on the changes in body burden of specific metal ions.

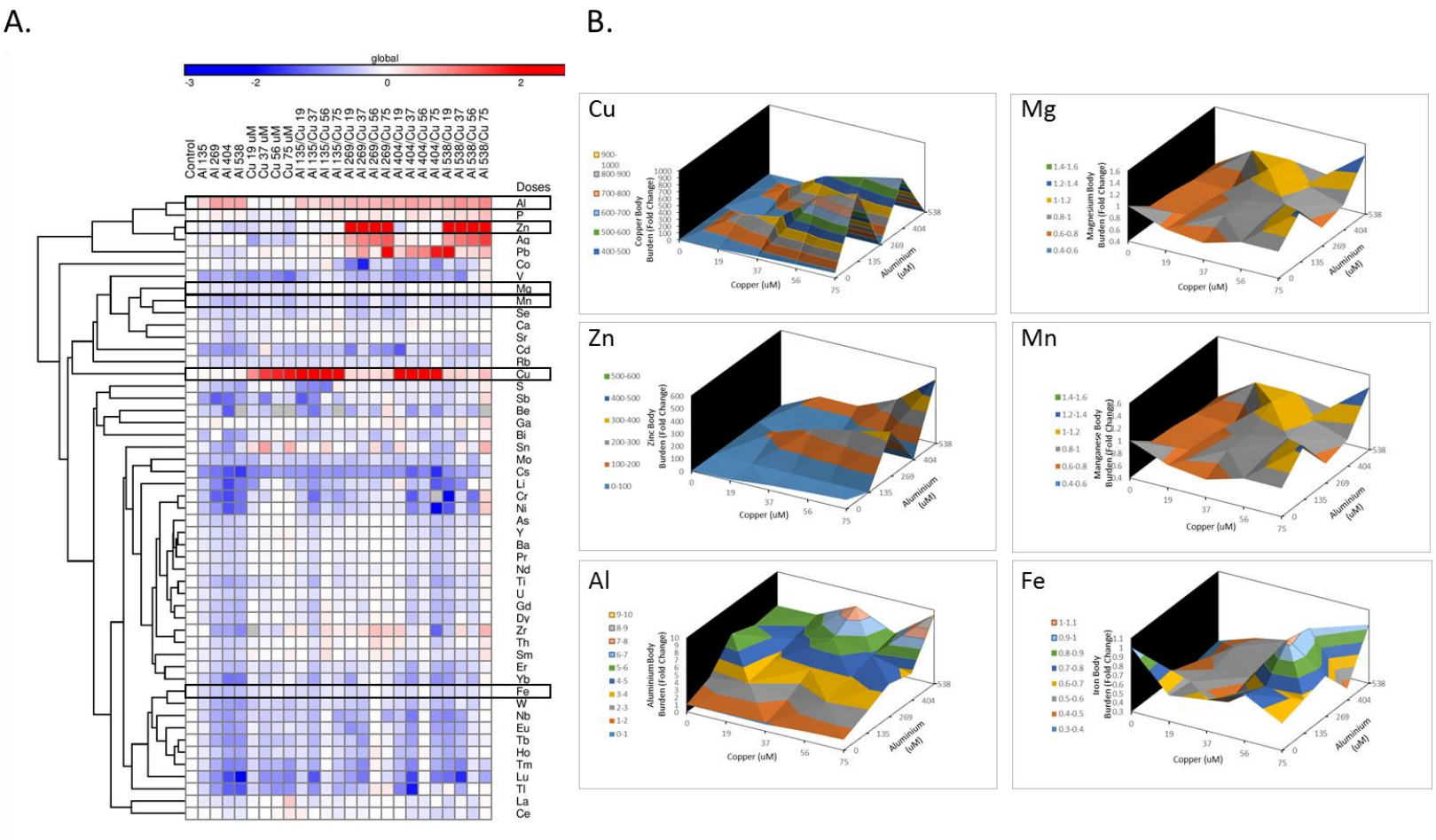

Figure 49: Changes in elemental composition of nematodes exposed to mixture of Copper and Aluminium. Nematodes were from age-synchronized cultures exposed to various concentrations of Copper and Aluminium. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 h period. Panel A: Hierarchical clustering of changes in ions levels in response to Cu/Al mixtures. Log2 (Mean fold change) is clustered for ion using a minus one Pearson correlation with average linage method applied. Panel B: Example topographic profiles for the impact of mixtures on the changes in body burden of specific metal ions.

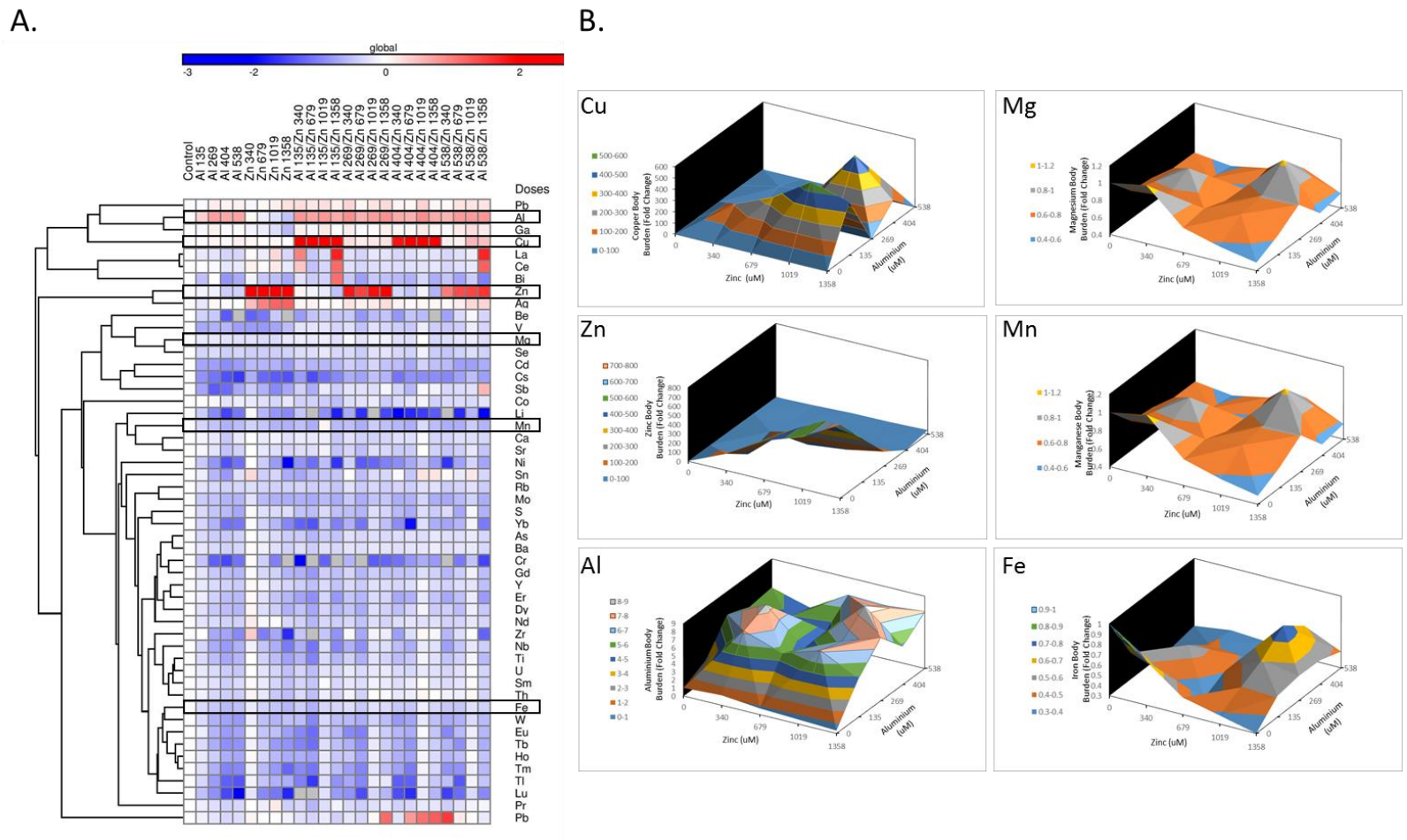

Figure 50: Changes in elemental composition of nematodes exposed to mixture of Zinc and Aluminium. Nematodes were from age-synchronized cultures exposed to various concentrations of Zinc and Aluminium. Biological replication was used to determined and average for each exposure, and at least five replicate exposures were conducted for each dose. Exposures were all performed for a 24 h period. Panel A: Hierarchical clustering of changes in ions levels in response to Zn/Al mixtures. Log2 (Mean fold change) is clustered for ion using a minus one Pearson correlation with average linage method applied. Panel B: Example topographic profiles for the impact of mixtures on the changes in body burden of specific metal ions.

8.3 DISCUSSION

For a better understanding of these metal mixture interactions along with the corresponding harmful effects they produce, we carried out more specific evaluations of the interactions of Zinc, Copper and Aluminium in binary mixtures. When comparing the effect of the metals mixtures on the mortality of *C. elegans*, it was demonstrated there was no significant effect ($p < 0.05$) of all different metals doses studied on the mortality of *C. elegans*. In general, all studied metal mixtures had a different level of reduction in the *C. elegans* survival tests. However, as mentioned previously, after statistical analysis, no change was found to be statistically significant. In the reproduction study, it was noticed that all mixtures had a significant reduction impact on the numbers of *C. elegans* eggs laid after the exposure. Noticeably, the most effect was found with the Copper/Aluminium and Zinc/Aluminium exposures tests, while Copper/Zinc mixtures was found to have the lowest impact.

The *C. elegans* metabolomics profile (amino acids) for Copper/Zinc mixture test showed that the interaction between Copper and Zinc had an impact on the amino acids profile. Most of the amino acids were elevated when compared to the control sample. Noticeably, Cysteine was the most affected amino acid in all our experiments, increasing with all Copper/Zinc mixtures tested, while Glycine and Glutamic acid were the most commonly decreased amino acids in some of our Copper/Zinc mixture tested. For the Copper/Aluminium mixture exposure, it was shown that the interaction between the three lowest Aluminium doses (LC_{10}, LC_{20} and LC_{30}) with all Copper doses decreased most of the amino acids in the nematode. On the other hand, the highest Aluminium dose (LC_{40}) when mixed with all the different Copper doses elevated nearly all amino acids in *C. elegans* with

the exception of Lysine which decreased. No exact (increasing or decreasing) trend was observed for the Zinc/Aluminium mixture test.

With regards to the *C. elegans* metabolomics profiles (fatty acids), the Copper/Zinc mixture noticeably decreased almost all fatty acids measured with the exception of a few specific ones such as (C20:0 in Zinc LC_{30} mixed with Copper LC_{40} and C20:4n3 in Zinc LC_{20} mixed with Copper LC_{20}). The same trend was shown in Copper/Aluminium and Zinc/Aluminium mixture test, in which nearly all fatty acids were decreased, except for C15:0 which rose in two exposures: the Aluminium doses LC_{30} and LC_{40} mixed with all Copper and Zinc sub-lethal doses. However, the Zinc/Aluminium mixture experiment demonstrated that the levels of some fatty acids increased slightly including: C14:0, C17:0, C18:0 and C20:0. The combined toxic effects of a binary mixture of Copper and Zinc were more harmful than the effects of individual elements, therefore a suggesting synergistic interaction between the two metals. According to the findings of Maazouzi *et al.*, (2008), the total fatty acid content of *D. villosus* decreased on chronic Copper exposure. According to our results, Copper and Zinc mixture exposures increased Glutamate and decreased fatty acids in *C. elegans*. Regarding the study by Taylor & Halperin (1975), Glutamate inhibited Glucose conversion to fatty acids in adipocytes from rats has been fed by Glutamate, and this inhibition was significantly tested relating to the Glutaminolyitc pathway Figure 51. In addition, Glutamate added to homogenates of adipose tissue had no effect on the activation of pyruvate dehydrogenase by Mg^{2+}. However, glutamate inhibited the active form of the enzyme and enhanced the rate of inactivation of the enzyme complex by ATP and Mg^{2+} (Taylor & Halperin, 1975), which was matched in our results.

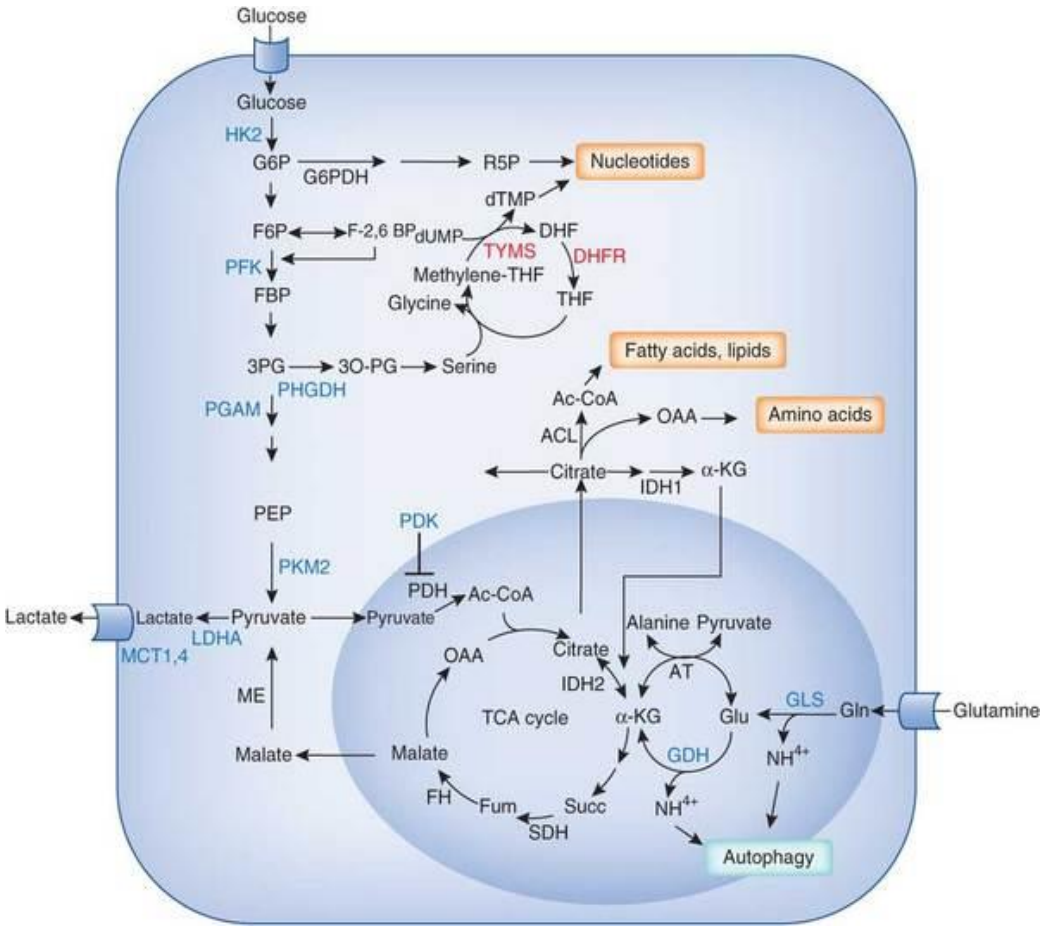

Figure 51: The core metabolic pathways and metabolic enzymes. Active metabolic pathways in proliferating cells involving Glucose and Glutamine catabolism are interconnected and linked to macromolecular synthesis and energy balance. Key metabolic enzymes discussed in the text (shown in blue) are actively investigated as therapeutic targets for cancer treatment (Cheong *et al.*, 2012).

According to the SPSS Automatic Linear Modelling Figure 52, we may categorizing the top 10 important predictors into two main classes (Positive and Negative) that relate to the different Copper and Zinc mixture exposures depending on the estimated coefficient as follows:

- Positive Predictors: C13:0, Copper, Tin, C22:0, Glutamine, Lead and C15:0.
- Negative Predictors: Lutetium, Aspartic Acid and Bismuth.

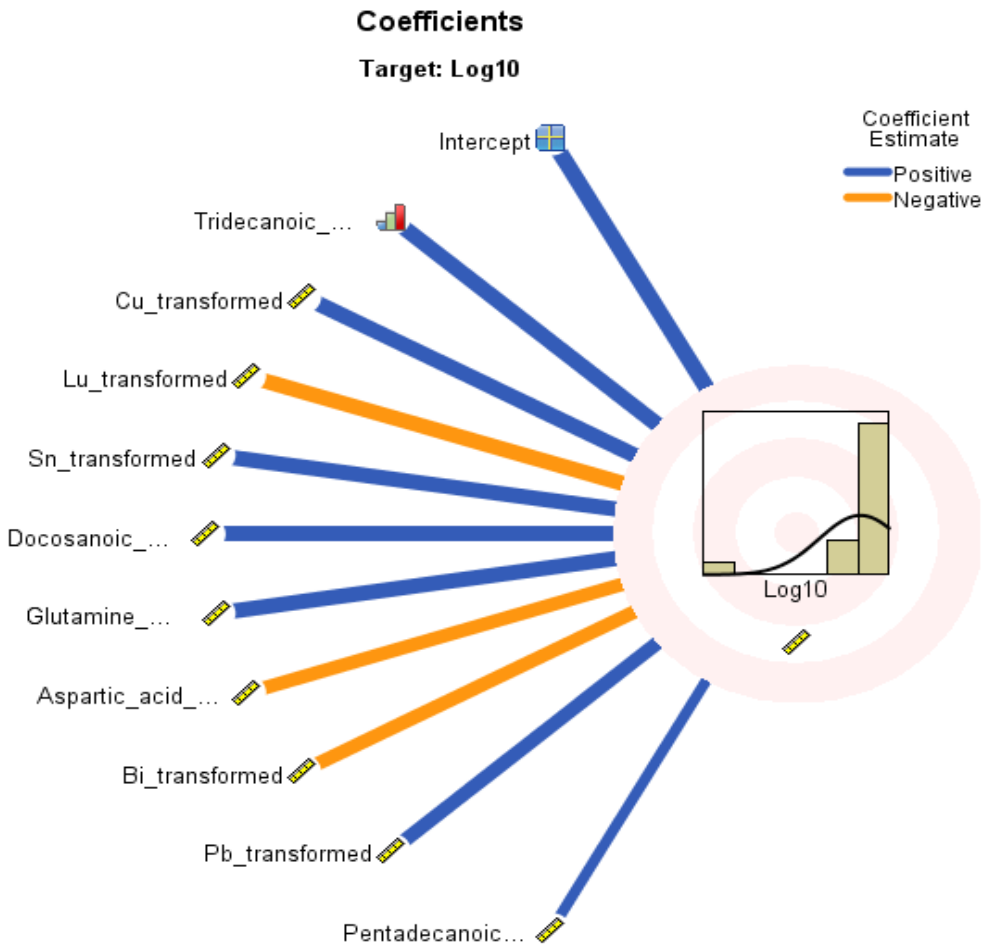

Figure 52: Coefficients (SPSS Linear models) for Copper and Zinc mixture. This chart displays the intercept first and then sorts effects from top to bottom in decreasing predictor importance. Within effect containing factors, coefficients are sorted by ascending order of data values. Connecting lines in the diagram are coloured based on the sign of the coefficient (Positive or Negative) and weighted based on coefficient significance, with greater line width corresponding to more significant coefficients (smaller *p*-values). The target is Copper and Zinc mixture exposures concentrations (Log 10).

Comparing results of the from the new SPSS feature (Automatic Linear Modelling) with the most acceptable statistic tests (ANOVA and t-test), could reject some of the predictors that did not match, such as: C13:0, C15: and C22:0 as these parameters decreased with Copper and Zinc mixture exposures.

According to the SPSS Automatic Linear Modelling Figure 53, we may categorizing the top 10 important predictors into two main classes (Positive and Negative) that related to the different Copper and Aluminium mixture exposures depending on the estimating coefficient as follow:

- Positive Predictors: C13:0, Copper, Silver, Chromium, Ornithine and Lanthanum.
- Negative Predictors: Cerium, Reproduction, Glycine and Nickle.

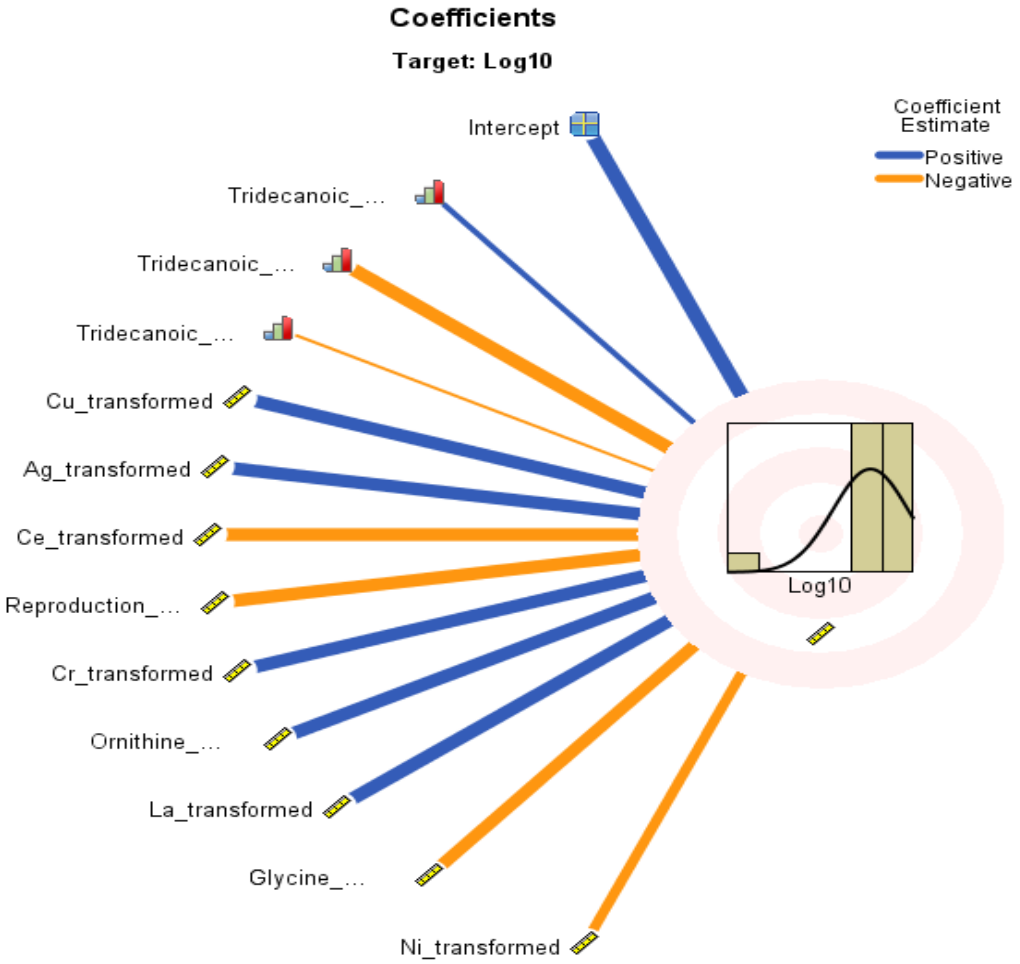

Figure 53: Coefficients (SPSS Linear models) for Copper and Aluminium mixture exposure. This chart displays the intercept first and then sorts effects from top to bottom in decreasing predictor importance. Within effect containing factors, coefficients are sorted by ascending order of data values. Connecting lines in the diagram are coloured based on the sign of the coefficient (Positive or Negative) and weighted based on coefficient significance, with greater line width corresponding to more significant coefficients (smaller *p*-values). The target is Copper and Aluminium mixture exposures concentrations (Log 10).

The study by Alexander *et al.,* (2008) showed that there were significant links between concentrations of ω-3 fatty acids in red blood cells and/or the concentration of ornithine in the plasma on the development of complications in renal transplant patients. However, he recommended that the relationship between concentrations of ω-3 and ω-6 fatty acids in red blood cell membranes and plasma ornithine might merit further study into the possibility of reducing complications after kidney transplant in humans receiving immuno-suppression treatment.

El-Hafidi *et al.,* (2004) reported in their study that Glycine, a nonessential amino acid, when administered in the diet, could be shown to be protective against the nonenzymatic haemoglobin Glycation (sometimes called non enzymatic Glycosylation, which is the result of covalent bonding of a protein or lipid molecules with a sugar molecule such as; Fructose or Glucose) found in diabetic humans and in diabetic rat models. Another group has attributed gelatine's lowering effect on plasma cholesterol and triglycerides (TGs) to its high glycine content (El-Hafidi *et al.,* 2004). This group showed that Gelatine, which contained 12 times more Glycine than Casein, decreased plasma cholesterol and TGs when administered in the diet of hypercholesterolemia rats.

According to the SPSS Automatic Linear Modelling Figure 54, we may categorizing the top 10 important predictors into two main classes (Positive and Negative) that related to the different Zinc and Aluminium mixture exposures depending on the estimated coefficient as follow:

- Positive Predictors: iso-C15:0, C17:0, Eu, Glutamic acid, C16:0 and Silver.
- Negative Predictors: Asparagine, C15:0, Tin, and C20:5n3.

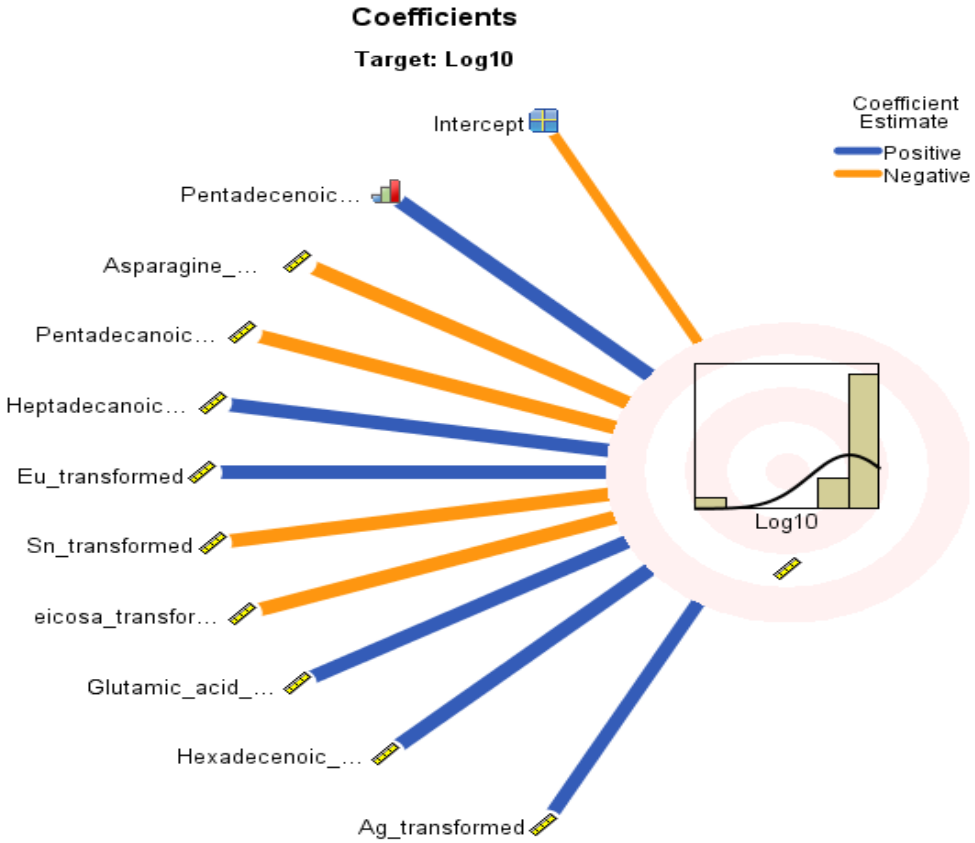

Figure 54: Coefficients (SPSS Linear models) for Zinc and Aluminium mixture. This chart displays the intercept first and then sorts effects from top to bottom in decreasing predictor importance. Within effect containing factors, coefficients are sorted by ascending order of data values. Connecting lines in the diagram are coloured based on the sign of the coefficient (Positive or Negative) and weighted based on coefficient significance, with greater line width corresponding to more significant coefficients (smaller *p*-values). The target is Zinc and Aluminium mixture exposures concentrations (Log 10).

Comparing results from the new SPSS feature (Automatic Linear Modelling) with the statistic tests (ANOVA and t-test), we could reject some of the predictors that do not match such as iso-C15:0, C17:0, as these parameters increased with Zinc and Aluminium mixture exposures, while Europium is increased with Zinc and Aluminium mixture exposures.

Nayak & Chatterjee (2001) reported that the glutamate level was noted to be increased significantly in all the tested brain regions of aluminium-treated rats. One of the contributory factors for this increase in glutamate levels may be aluminium-induced inhibition of Glutamate transport by the synaptic vesicles or the inhibition of Glutamate release by Aluminium. Glutamate levels in the brain may also be modulated by its altered metabolism. Several Glutamate metabolizing enzymes may be involved in the altered metabolism of Glutamate leading to modulation of its concentration. Eder & Kirchgessner (1996) observed no change or even an increase of the other desaturase metabolites as Eicosapentaenoic acid (20:5n-3), 22:4n-6 and 22:5n-6 (Eder *et al.* 1994). Wang *et al.*, (2003) concluded in their study that Zn^{2+} inhibits the activity of fatty acid synthase.

Possible explanations for some of these differences in results reported by various investigators may be the different rates of absorption of the metals for different concentrations and environmental test conditions, differences in the physiological condition of test organisms, and differences in models hypothesized and interpretation of results. From the results, it was obvious that no clear trends emerged that would permit a simple generalization. No simple correlations were obtained that could relate the toxic effect of binary mixtures to those of individual metals. A better understanding of dose responses interactions is needed for achieving predictive capability for toxic effect of mixtures.

9 DISCUSSION

9.1 RED SEA SAMPLES

Analysis of the metal composition of the water samples collected at the off-shore sampling stations indicated that Copper was the highest represented metal, followed by Arsenic and Nickle. Lead was the least abundant metal. In addition, in our near-shore samples the Copper was further elevated, while Cadmium was not detected. For Manganese and Zinc, values were much higher in 1994, whilst in 2006 they had fallen which reported by Massoud & Fahmy, (1994) and El-Ghazaly & Abdel-Aziz, (2006). Our results determined that in the summer of 2011, Manganese to have again risen and exceeded its 1994 level, while no Zinc was detected, which may due to the detection limit of the ICP-MS used. Similarly, Lead which was measured in 2006 has been recorded approximately 14 fold higher. Studies over the last 20 years have confirmed the observation that heavy metals are rising in the Red Sea waters especially in near-shore water samples as a result of human activities.

According to the sediment samples collected from the off-shore water stations at a depth of about 65 meters, Aluminium has been the most abundant metal closely followed by Lead, Arsenic, Nickle and Copper, whilst other metals were found at trace levels. Soils are considered as sinks for trace elements; therefore, they play an important role in environmental cycling of these elements. They have a great ability to fix many species of trace ions and every soil component is active and affects soil solution ion concentration either by precipitation dissolution reactions or by ionic interactions with phase surfaces (Shahidul Islam & Tanaka, 2004). Plankton samples collected both from near-shore and off-shore sampling stations did not yield measurements above the detection limit of the ICP-MS

instrument. Hydrocarbons were below detectable limits in sea water, sediments, plankton and fish samples, which reflects the Gas-Chromatography instrument detection limit.

Our collection of a local fish species, the spangled emperor *Lethrinus nebulosus*, was successful and revealed intriguing relationships with some of the metals, especially Aluminium and Zinc, recorded in associated sediments samples. *L. nebulosus* inhabits depths of between 10 and 75 metres and it is a non-migratory species found on coral and rocky reefs, sea grass beds, mangrove swamps, as well as over sandy substrates (Drummond, 1996). In our collected samples of Lethrinus nebulosus in 2011, Aluminium and Zinc were observed to be the metals present in the highest concentration in fish tissues (gill and liver). These differences may related to the collection sites and the ecology of the fish species. The elevation of Aluminium in both the gills and liver tissues, is intriguing especially given the low levels observed in the sea water and the high levels recorded in the sediments. It is possible that the Molluscs, Echinoderms, Crustaceans and Polychaetes that form the majority of its diet may provide the basis for the transfer from sediment to the fish but it was not possible to test this hypothesis within the context of the current study. Fish are extensively used to assess the condition of aquatic systems as contaminants accumulate in the food chain and cause deleterious effects and ultimately death within the aquatic system (Vinodhini and Narayanan, 2008).

9.2 COPPER EXPOSURES TESTS

Our mortality and reproduction data revealed that this impairment could be due to a reduction in the amount of metabolic energy available. For amino acids analysis that we have noticed that Cystathionine, Cysteine and Methionine have been rose. These amino acids are

containing a thiol group in their structures, which is most likely to interact with Copper regarding the chemical affinity between these two ions (Copper and Sulphur). For fatty acids analysis, we have noticed generally that Copper has reduced most of the fatty acids. Copper is a superoxide generator, and can induce oxidative stress in tissues. It has ability to induce the formation of hydrogen peroxide (H_2O_2), which can be transformed to hydroxyl radical OH, which is extremely reactive and modifies all biomolecules in its vicinity, including lipids, proteins and DNA (Prato *et al.,* 2013). Cystathionine is the key intermediate in the trans-sulfuration pathway, a reaction by which Cysteine and Methionine can be generated (Rao *et al.,* 2005). It is an important pathway as it is the only process by which cysteine, a non-essential amino acid is generated and requires the use of the essential amino acid methionine (Houghton & Cherian, 1991). This pathway is important for generating phytochelatin synthase which is required for protection against some toxic metals. Metallothioneins are believed to play role in organism's responses to and protection against toxic metal ions. According to Maazouzi *et al.,* (2008) chronic Copper exposure affects *D. villosus* decreased its total fatty acids content. The high affinity Copper uptake protein Ctr1 is responsible for making Copper that enters via the apical membrane available in the cytosol for further utilization, whereas the Copper-transporting ATPase ATP7A facilitates Copper exit from the enterocytes into circulation.

9.3 ZINC EXPOSURES TESTS

Our results suggest that when *C. elegans* is exposed to >1 mM Zinc for 24 hours, survival and reproduction have been decreased. In our experiments, sub-lethal exposure of *C. elegans* to Zinc caused a general increase in most metabolites (Amino Acids). Our findings

have showed that Zinc has a remarkable effect on the nematode fatty acid profile, decreased the amount of all fatty acids. Our Ionomics results showed that Zinc exposure had a significant impact on internal elemental balance in *C. elegans* include increased amounts of Silver and Phosphorus; however, the majority of the remaining ions decreased. Recent data reinforces Zinc's essential role, confirming its widespread involvement in development, immunity, reproduction, endocrinology and neurotransmission (Hogstrand *et al.,* 2009). It was also shown that in the presence of Zinc, reproduction was considerably 'slowed', egg laying frequency reduced, and at higher concentration of Zinc, reproduction was fully inhibited (Bruinsma *et al.,* 2008; Chu *et al.,* 2002).

Zinc acts as a cofactor for an estimated 3,000 human proteins, representing 10% of the proteome and has a well-established role in regulation of gene expression through metal-responsive transcription-factor-1 (MTF1) (Hogstrand *et al.,* 2008). Zinc binding domains are typically characterized by Zinc (II) bound in a tetrahedral geometry to four protein derived ligands, most frequently nitrogen from Histidine, Sulphur from Cysteine and oxygen from aspartate or glutamate. Zinc induces the synthesis of metallothioneins which is a factor in regulating the metabolism of Zinc including absorption and storage (Miles *et al.,* 2000). In opinion of Ribeyre *et al.,* (1995) bioaccumulation of Silver is greatly affected by the amount of Zinc present in water, the combined action of the elements responsible for increased amounts of Silver in the organisms. Metallothionein (MT) is a small cysteine-rich, intracellular protein that avidly binds metals of groups IB and IIB of the Periodic System In mammalian cells in vitro, Silver seems to be one of the more potent inducers of MT (Hogstrand, 1996).

9.4 ALUMINIUM EXPOSURES TESTS

Aluminium has a modest impact on mortality with survival rates of ~80%. Aluminium exposure clearly has reduced *C. elegans* fertility. At the lower dose (135 µM), many of the amino acid constituents are significantly reduced with the exception of Aspartic acid, Hydroxylysine, Ornithine and Tryptophan. Significant reductions in a range of amino acids are also seen when nematodes are exposed to (269 and 404 µM) Aluminium but interestingly this impact is reversed at (538 µM) where many of the same amino acids are seen to increase. Our fatty acids experiments showed an expected result with Aluminium, as it has caused a general decrease in most of the fatty acids measured with the exception of C14:0 and C20:4n3 which were observed to be increased under the lowest Aluminium exposure. Our results have shown that Aluminium caused reduction to most of the elemental profiles of whole nematodes. Detailed biochemical studies have indicated that Aluminium exerts strong influence the activity of many enzymes. Aluminium forms tight complexes with ATP so it is a strong inhibitor of numerous enzymes utilising ATP as a substrate. The following enzymes are also inhibited by Aluminium: Hexokinase, Alkalic Phosphatase, Choline acetyltransferase, Ferrooxidase (Zaman *et al.*, 1993; Barabasz *et al.*, 2002). Several studies have shown that oxidative stress induced by Aluminium modifies the peroxidation of lipids and the activities of anti-oxidative enzymes and identified increased peroxidation of lipids in brain tissues of adult *Wistar* rats following administration of Aluminium intake (Zaman *et al.*, 1993; Becaria *et al.*, 2002; Ondreička *et al.*, 1966). Aluminium toxicity may result from the fact that Aluminium has higher binding affinities compared to the physiological binding partners, including the replacement of Zn^{2+}, Mg^{2+} and Fe^{3+} by Al^{3+} in key metallo-enzyme (Yang, 1998). It is our proposition that high levels of Aluminium influence metallostasis by causing changes in the trace element pool and that this disturbance

results in an inequality in elemental concentrations and stimulates the production of oxidative stress.

9.5 MIXTURES TESTS

In generally, all studied metal mixtures have a different level of reduction impact on the *C. elegans* survival tests. Noticeably, the most effect have been found with the Copper/Aluminium and Zinc/Aluminium exposures tests, while Copper/Zinc mixtures has been found the lowest impact. The interaction between Copper and Zinc has an impact on the amino acids profile. Most of the amino acids have been elevated in amount comparing with control sample. Noticeably, Cysteine has been the most increased affected amino acid in all our designed treatment. Copper/Aluminium mixture test shows that the interaction have been decreased most of the amino acids in the nematode. No exact trend has been shown for the Zinc/Aluminium mixture test. Although, most of amino acids have been decreased regarding this treatment except some amino acids have been increased in some mixture exposures. With regards to the *C. elegans* metabolomics profile (fatty acids), the all mixtures noticeably decreased almost most of fatty acids measured with the exception of a few ones. Ionomics profile of *C. elegans* data has shown the impact of Copper/Zinc mixture on different metals in *C. elegans*. Generally, all the elements have been decreased in the all mixtures designed except the Silver has been increased in all designed exposures. The same trend has been shown for Copper/Aluminium mixtures, all elements have been decreased except the Phosphorus and Zinc have been increased. For Zinc/Aluminium mixtures, data has shown also dropped in most of the element profile except Copper and Phosphorus were increased in most of designed exposures.

Copper and Zinc work together to support your metabolism. Both minerals help to activate the enzyme copper-zinc superoxide dismutase, also called Cu/Zn-SOD. This enzyme serves as an antioxidant, which means that it protects your cells from harmful reactive oxygen species, a group of chemicals that form as a natural by-product of your cell's metabolism. Since your cells constantly produce new reactive oxygen species, they rely on antioxidants to continually neutralize the compounds and prevent cell damage. Getting enough Copper and Zinc helps ensure that you can effectively clear away reactive oxygen species, so that your cells can continue to function properly. The combined toxic effects of a binary mixture of Copper and Zinc were more harmful than the effects of individual elements suggesting synergistic interactions between the two metals. According to the findings of Maazouzi *et al.,* (2008), the total fatty acid content of *D. villosus* decreased on chronic copper exposure. According to our results, Copper and Zinc mixture exposures increased Glutamate and decreased fatty acids in *C. elegans*. Regarding to the study by Taylor & Halperin, (1975), Glutamate inhibited Glucose conversion to fatty acids and this inhibition was significantly tested relating to the Glutaminolyitc pathway. The variation in the experimental results of different researchers can possibly be explained in terms of different rates of absorption of the metals for different concentrations and environmental test conditions, variations in the physiological condition of test organisms, and inconsistencies in models theorized and analysis of results. In reality, many factors are simultaneously regulating and complicating the projection of the effects of mixtures of toxicants. To precisely predict and project the toxic effect of mixture requires a thorough understanding of metal responses interactions and genomic studies may considered.

10 CONCLUSION

Biomarkers are mostly useful in the evaluation of progressive diseases that apparent their symptoms long after exposure to the initiating factor. In such cases, traditional early warning symptoms of developing disease may be lacking. It is important to identify and address the growing environmental problems being faced by the community and address it before it takes the shape of an epidemic.

From the results, it is obvious that no clear trends have emerged that would permit a simple generalization. Indeed, several factors influence and complicate prediction of the effects of mixtures of toxicants. To gain a full system analysis it would be essential to performed global analysis of change sin gene expression (transcriptomic) to complement the current work or at minimum assess the transcript and protein levels of key biosynthetic/catabolic enzymes, transporters and metal binding proteins, such as Metallothionein. Furthermore, a better understanding of the temporal responses underlying the interactions is needed for achieving predictive capability for toxic effect of mixtures.

11 REFERENCES

Ahmed, S. & Santosh, W. (2010). Metallomic profiling and linkage map analysis of early Parkinson's disease: a new insight to aluminum marker for the possible diagnosis. PLoS One, 5, 11252.

Al-Bader, N. (2008). Heavy metal levels in most common available fish species in Saudi Market. J. Food Technol., 6, 173–177.

Aleem, a. a. (1990). Impact of human activity on marine habitats along the Red Sea coast of Saudi Arabia. Hydrobiologia, 208, 7–15.

Alexander, J.W., Goodman, H.R., Succop, P., Light, J.A., Kuo, P.C., Moser, A.B., et al. (2008). Influence of long chain polyunsaturated fatty acids and ornithine concentrations on complications after renal transplant. Exp. Clin. Transplant., 6, 118–126.

Al-Farawati, R. (2010). Environmental conditions of the coastal waters of Southern Corinche, Jeddah, Eastern red sea: Physico-chemical approach. Aust. J. Basic Appl. Sci, 4, 3324–3337.

Almohanna, M.M. (2000). Determination of Aromatic Hydrocarbons in the Gill Extract of Five Different Fish Species Along the Red Sea Coast of Jeddah and Gizan. KAU, 11, 3–17.

Altenburger, R. (2010). Understanding combined effects for metal co-exposure in ecotoxicology. Met. Ions Life Sci., 8, 1–26.

Anderson, G.L., Boyd, W. a & Williams, P.L. (2001). Assessment of sublethal endpoints for toxicity testing with the nematode Caenorhabditis elegans. Environ. Toxicol. Chem., 20, 833–8.

Aruoma, O., Halliwell, B., Gajewski, E. & Dizdaroglu, M. (1991). Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. Biochem. J, 273, 601–604.

Aspholm, O.Ã. & Hylland, K. (1998). Metallothionein in green sea urchins (Strongylocentrotus droebachiensis) as a biomarker for metal exposure. Mar. Environ. Res., 46, 537–540.

Atherton, H., Jones, O., Malik, S., Miska, E. & Griffin, J. (2008). A comparative metabolomic study of NHR-49 in Caenorhabditis elegans and PPAR-α in the mouse. FEBS Lett., 582, 1661–1666.

ATSDR. (2001). Toxicological profile for aluminum. Agency for Toxic Substance and Disease Registry; U.S. Department of Health and Human Services.

Baas, J., Van Houte, B.P.P., Van Gestel, C. a M. & Kooijman, S. a L.M. (2007). Modeling the effects of binary mixtures on survival in time. Environ. Toxicol. Chem., 26, 1320–1327.

Baldwin, J., Avila, D.S., Adams, M.R., Chakraborty, S. & Aschner, M. (2011). Reproductive and developmental toxicity. Reprod. Dev. Toxicol., 193–205.

Barabasz, W., Albinska, D., Jaskowska, M. & Lipiec, J. (2002). Ecotoxicology of aluminium. Polish J. Environ. Stud., 11, 199–203.

Baxter, I. (2009). Ionomics: studying the social network of mineral nutrients. Curr. Opin. Plant Biol., 12, 381–386.

Baxter, I. (2010). Ionomics: The functional genomics of elements. Brief. Funct. Genomics, 9, 149–56.

Baxter, I.R., Vitek, O., Lahner, B., Muthukumar, B., Borghi, M., Morrissey, J., et al. (2008). The leaf ionome as a multivariable system to detect a plant's physiological status. Proc. Natl. Acad. Sci. U. S. A., 105, 12081–12086.

Becaria, a, Campbell, A. & Bondy, S.C. (2002). Aluminum as a toxicant. Toxicol. Ind. Health, 18, 309–320.

Bertini, I. & Cavallaro, G. (2008). Metals in the "omics" world: copper homeostasis and cytochrome C oxidase assembly in a new light. J. Biol. Inorg. Chem., 13, 3–14.

Beyersmann, D. & Hartwig, A. (2008). Carcinogenic metal compounds: Recent insight into molecular and cellular mechanisms. Arch. Toxicol.

Bliss, C.I. (1939). The toxicity of poisons applied jointly. Ann. Appl. Biol., 26, 585–615.

Booth, S.C., Workentine, M.L., Weljie, A.M. & Turner, R.J. (2011). Metabolomics and its application to studying metal toxicity. Metallomics, 1142–1152.

Borgmann, U. (2008). Modelling bioaccumulation and toxicity of metal mixtures. Hum. Ecol. Risk Assess. An Int. J., 14, 266–289.

Boyle, D., Hogstrand, C. & Bury, N.R. (2011). Physiological response to a metal-contaminated invertebrate diet in zebrafish: importance of metal speciation and regulation of metal transport pathways. Aquat. Toxicol., 105, 21–28.

Braeckman, B.P., Houthoofd, K. & Vanfleteren, J.R. (2009). Intermediary metabolism. WormBook, 1–24.

Breaux, H.J. (1968). A modification of Efroymson's technique for stepwise regression analysis. Commun. ACM, 11, 556–558.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics, 77, 71–94.

Bridges, C.C., Zalpus, R.K. & Zalups, R.K. (2005). Molecular and ionic mimicry and the transport of toxic metals. Toxicol Appl Pharmacol, 204, 274–308.

Bruinsma, J.J., Schneider, D.L., Davis, D.E. & Kornfeld, K. (2008). Identification of mutations in Caenorhabditis elegans that cause resistance to high levels of dietary zinc and analysis using a genomewide map of single nucleotide polymorphisms scored by pyrosequencing. Genetics, 179, 811–828.

Bundy, J.G. & Kille, P. (2014). Metabolites and metals in Metazoa--what role do phytochelatins play in animals? Metallomics, 6, 1576–82.

Bundy, J.G., Sidhu, J.K., Rana, F., Spurgeon, D.J., Svendsen, C., Wren, J.F., et al. (2008). "Systems toxicology" approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm Lumbricus rubellus. BMC Biol., 6, 25.

Campbell, P.G.C.P., Errécalde, O., Fortin, C., Hiriart-Baer, V.P. & Vigneault, B. (2002). Metal bioavailability to phytoplankton applicability of the biotic ligand model. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. Physiol. Part C, 133, 189–206.

Cassee, F. & Groten, J. (1998). Toxicological evaluation and risk assessment of chemical mixtures. CRC Crit. Rev., 1–50.

Cheong, H., Lu, C., Lindsten, T. & Thompson, C.B. (2012). Therapeutic targets in cancer cell metabolism and autophagy. Nat. Biotechnol.

Chu, K.W., Chow, K.L. & Wah Chu, K. (2002). Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. Aquat. Toxicol., 61, 53–64.

Chung, N. J., Walker, P. A., Brown, R. W. and Hogstrand, C. (2005). ZINC-mediated gene expression offers protection against H2O2-induced cytotoxicity. *Toxicology and Applied Pharmacology* 205, 225-236.

Cooper, N.L., Bidwell, J.R. & Kumar, A. (2009). Toxicity of copper, lead, and zinc mixtures to Ceriodaphnia dubia and Daphnia carinata. Ecotoxicol. Environ. Saf., 72, 1523–1528.

Cousins, R.J., Liuzzi, J.P. & Lichten, L.A. (2006). Mammalian zinc transport, trafficking, and signals. J. Biol. Chem.

Cunnane, S.C., Horrobin, D.F. & Manku, M.S. (1984). Essential fatty acids in tissue phospholipids and triglycerides of the zinc-deficient rat. Proc. Soc. Exp. Biol. Med., 177, 441–446.

Dhawan, R. & Dusenbery, D. (2000). A comparison of metal-induced lethality and behavioral responses in the nematode Caenorhabditis elegans. Toxicol. Chem., 19, 3061–3067.

Dhawan, R., Dusenbery, D.B. & Williams, P.L. (1999). Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode Caenorhabditis elegans. J. Toxicol. Environ. Heal. Part A, 58, 451–462.

Dietz, R. & Johansen., F. riget and P. (1996). Lead, cadmium, mercury and selenium in Greenland marine animals. Sci. Total Environ. 186(1-2)67-93. 1996., 27.

Disease, H. (2008). Assessment of locomotion behavioral defects induced by acute toxicity from heavy metal exposure in nematode Caenorhabdities elegans, 20, 1132–1137.

Dixon, R.L., Sherins, R.J. & Lee, P. (2000). Assessment of Environmental Factors Affecting Male Fertility, 30, 53–68.

Donkin, S. & Dusenbery, D. (1994). Using the Caenorhabditis elegans soil toxicity test to identify factors affecting toxicity of four metal ions in intact soil. Water Air Soil Pollut., 78, 359–373.

Drummond, A. (1996). Zoological Catalogue of Australia Vol. 33 Echinodermata/F.W.E. Rowe & J. Gates. African Zool., 31.

Dudka, S. & Adriano, D.C. (1997). Environmental Impacts of Metal Ore Mining and Processing: A Review. J. Environ. Qual.

Duffus, B.J.H., Templeton, D.M., Nordberg, M. & Duffus, J.H. (2009). Concept Group 2 . Concepts Applying to Molecular and Cellular Toxicology, 52–99.

Dupont, C.L., Butcher, A., Valas, R.E., Bourne, P.E. & Caetano-Anollés, G. (2010). History of biological metal utilization inferred through phylogenomic analysis of protein structures. Proc. Natl. Acad. Sci. U. S. A., 107, 10567–10572.

Eder, K., Kirchgessher, M. & Universitã, D.T. (1994). Biochemical and Molecular Roles of Nutrients Dietary Fat Influences the Effect of Zinc Deficiency on Liver Lipids and Fatty Acids in Rats Force-Fed Equal Quantities of Diet1 ’ 2, 1917–1926.

Eder, K. & Kirchgessner, M. (1996). Zinc deficiency and the desaturation of linoleic acid in rats force-fed fat-free diets. Biol. Trace Elem. Res., 54, 173–183.

Eisler, R. (1998). Copper hazards to fish, wildlife, and invertebrates: A synoptic review.

El-ghazaly, N. (2006). In coastal water of Jeddah on the 1-Histological structure of gills and intestine of the fish Siganus Rivulatus (forskal). Egypt. J. Aquat. Res., 32, 298–315.

El-Hafidi, M., Pérez, I., Zamora, J., Soto, V., Carvajal-Sandoval, G. & Baños, G. (2004). Glycine intake decreases plasma free fatty acids, adipose cell size, and blood pressure in sucrose-fed rats. Am. J. Physiol. Regul. Integr. Comp. Physiol., 287, 1387–1393.

Equence, C.E.S. & Iology, T.O.B. (1998). Genome Sequence of the nematode C. elegans: A Platform for Investigating Biology. Science (80-.)., 282, 2012–2018.

Evans, C.W., Hills, J.M. & Dickson, J.M.J. (2000). Heavy metal pollution in Antarctica: a molecular ecotoxicological approach to exposure assessment. J Fish Biol, 57, 8–19.

Evstatiev, R. & Gasche, C. (2012). Iron sensing and signalling. Gut, 61, 933–52.

Fosmire, G. (1990). Zinc toxicity. Am. J. Clin. Nutr., 225–227.

Fox, J. (1997). Applied Regression Analysis, Linear Models, and Related Methods. Appl. Regres. Anal. Subsequent. SAGE Publications, Inc, London.

Fox, J. & Monette, G. (1992). Generalized Collinearity Diagnostics. J. Am. Stat. Assoc., 87, 178–183.

Fraga, C.G. (2005). Relevance, essentiality and toxicity of trace elements in human health. Mol. Aspects Med., 26, 235–44.

Ganrot, P.O. (1986). Metabolism and Possible Health Effects of Aluminum. Environ. Health Perspect., 65, 363.

Geier, F.M., Want, E.J., Leroi, A.M. & Bundy, J.G. (2011). Cross-platform comparison of Caenorhabditis elegans tissue extraction strategies for comprehensive metabolome coverage. Anal. Chem., 83, 3730–3736.

Girard, L.R., Fiedler, T.J., Harris, T.W., Carvalho, F., Antoshechkin, I., Han, M., et al. (2007). WormBook: The online review of Caenorhabditis elegans biology. Nucleic Acids Res.

Glover, C.N., Bury, N.R. & Hogstrand, C. (2004). Intestinal zinc uptake in freshwater rainbow trout: Evidence for apical pathways associated with potassium efflux and modified by calcium. Biochim. Biophys. Acta - Biomembr., 1663, 214–221.

Halliwell and Gutteridge. (2007). Free Radicals in Biology and Medicine. 4th edn. Oxford University Press.

Hamilton, S. & Mehrle, P. (1986). Metallothionein in fish: Review of its importance in assessing stress from metal contaminants. Trans. Am. Fish. Soc., 115, 596–609.

Hanas, J., Larabee, J. & Hocker, J. (2005). Zinc finger interactions with metals and other small molecules. Zinc Finger Proteins.

Hansen, C.T., Nielsen, C.O., Dietz, R. & Hansen, M.M. (1990). Zinc, cadmium, mercury and selenium in minke whales, belugas and narwhals from West Greenland (Arctic Ocean). Polar Biol., 10, 529–540.

Hitchcock, D.R., Black, M.C. & Williams, P.L. (1997). Investigations into using the nematode Caenorhabditis elegans for municipal and industrial wastewater toxicity testing. Arch. Environ. Contam. Toxicol., 33, 252–260.

Hogstrand, C., Kille, P., Nicholson, R.I. & Taylor, K.M. (2009). Zinc transporters and cancer: a potential role for ZIP7 as a hub for tyrosine kinase activation. Trends Mol. Med., 15, 101–111.

Hogstrand, C., Verbost, P.M., Bonga, S.E. & Wood, C.M. (1996). Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am. J. Physiol., 270, R1141–R1147.

Hogstrand, C., Zheng, D., Feeney, G., Cunningham, P. & Kille, P. (2008). Zinc-controlled gene expression by metal-regulatory transcription factor 1 (MTF1) in a model vertebrate, the zebrafish. Biochem. Soc. Trans., 36, 1252–1257.

Houghton, C.B. & Cherian, M.G. (1991). Effects of inhibition of cystathionase activity on glutathione and metallothionein levels in the adult rat. J. Biochem. Toxicol., 6, 221–228.

Howell, R. (1983). Heavy metals in marine nematodes: Uptake, tissue distribution and loss of copper and zinc. Mar. Pollut. Bull., 14, 263–268.

Hughes, S., Bundy, J. & Want, E. (2009). The metabolomic responses of Caenorhabditis elegans to cadmium are largely independent of metallothionein status, but dominated by changes in cystathionine and. J. Proteome Res., 3512–3519.

Hughes, S. & Stürzenbaum, S.R. (2007). Single and double metallothionein knockout in the nematode C. elegans reveals cadmium dependent and independent toxic effects on life history traits. Environ. Pollut., 145, 395–400.

Hulme, S.E. & Whitesides, G.M. (2011). Chemistry and the worm: Caenorhabditis elegans as a platform for integrating chemical and biological research. Angew. Chem. Int. Ed. Engl., 50, 4774–807.

Hurvich, C. & Tsai, C. (1989). Regression and time series model selection in small samples. Biometrika, 76, 297–307.

Huster, D., Purnat, T. D., Burkhead, J. L., Ralle, M., Fiehn, O., Stuckert, F., Olson, N. E., Teupser, D. and Lutsenko, S. (2007). High copper selectively alters lipid metabolism and cell cycle machinery in the mouse model of Wilson disease. *J Biol Chem* 282, 8343-55.

Jackson, K. a, Valentine, R. a, Coneyworth, L.J., Mathers, J.C. & Ford, D. (2008). Mechanisms of mammalian zinc-regulated gene expression. Biochem. Soc. Trans., 36, 1262–6.

Jeppe, K.J., Carew, M.E., Long, S.M., Lee, S.F., Pettigrove, V. & Hoffmann, A.A. (2014). Genes involved in cysteine metabolism of Chironomus tepperi are regulated differently by copper and by cadmium. Comp. Biochem. Physiol. - C Toxicol. Pharmacol., 162, 1–6.

Jomova, K. & Valko, M. (2011). Advances in metal-induced oxidative stress and human disease. Toxicology, 283, 65–87.

Jonker, M.J., Piskiewicz, A.M., Ivorra i Castellà, N. & Kammenga, J.E. (2004). Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode Caenorhabditis elegans. Environ. Toxicol. Chem., 23, 1529–1537.

Jonker, M.J.M.J., Svendsen, C., Bedaux, J.J.M.J.J.M., Bongers, M. & Kammenga, J.E.J.E. (2005). Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ. Toxicol. Chem., 24, 2701–2713.

Jytte Molin, C. (1995). Human exposure to toxic metals: factors influencing interpretation of biomonotoring results. Sci Total Env., 166, 89–135.

Kabata-Pendias, H.P. and A. (2000). Trace Elements in Soils and Plants. 3rd edn. CRC Press.

Kakkar, P. & Jaffery, F.N. (2005). Biological markers for metal toxicity. Environ. Toxicol. Pharmacol., 19, 335–349.

Kaletta, T. & Hengartner, M.O. (2006). Finding function in novel targets: C. elegans as a model organism. Nat. Rev. Drug Discov., 5, 387–398.

Kalis, E. & Temminghoff, E. (2006). Effects of humic acid and competing cations on metal uptake by Lolium perenne. Environ. Toxicol. Chem., 25, 702–711.

Kanumakala, S., Boneh, A. & Zacharin, M. (2002). Pamidronate treatment improves bone mineral density in children with Menkes disease. J. Inherit. Metab. Dis., 25, 391–398.

Kebeish, R., El-ayouty, Y. & Husain, A. (2014). Effect of copper on growth bioactive metabolites antioxidant enzymes and photosynthesis related gene transcription in Chlorella vulgaris, 2, 34–43.

Kenyon, C. & Sulston, J. and Hodgkin, J. (1988). The Nematode Caenorhabditis elegans. Science (80-.). Cold Spring Harbor Laboratory Press, New York.

Khanna, N., Cressman, C.P., Tatara, C.P. & Williams, P.L. (1997). Tolerance of the nematode Caenorhabditis elegans to pH, salinity, and hardness in aquatic media. Arch. Environ. Contam. Toxicol., 32, 110–114.

Kinraide, T. (1999). Interactions among Ca2+, Na+ and K+ in salinity toxicity: quantitative resolution of multiple toxic and ameliorative effects. J. Exp. Bot., 50, 1495–1505.

Kito, H., Ose, Y., Mizuhira, V. & Sato, T. (1982). Separation and purification (cd , cu , zn) - metallothionein in carp hepato-pancreas. Comp. Biochem. Physiol., 73, 121–127.

Kniazeva, M., Crawford, Q.T., Seiber, M., Wang, C.Y. & Han, M. (2004). Monomethyl branched-chain fatty acids play an essential role in Caenorhabditis elegans development. PLoS Biol., 2.

Komjarova, I. & Blust, R. (2008). Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in water flea Daphnia magna, a stable isotope experiment. Aquat. Toxicol., 90, 138–144.

Kramer, T.I.M.R., Rriske-anderson, M., Susan, B. & Holman, J.A.T. (n.d.). Influence of Reduced Food Intake on Polyunsaturated Fatty Acid Metabolism in Zinc-Deficient Rats1, 1224–1230.

Kühnl, J., Bobik, T., Procter, J.B., Burmeister, C., Höppner, J., Wilde, I., et al. (2005). Functional analysis of the methylmalonyl-CoA epimerase from Caenorhabditis elegans. FEBS J., 272, 1465–1477.

Lee, J., Peña, M.M.O., Nose, Y. & Thiele, D.J. (2002). Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem., 277, 4380–4387.

Leggat, P.A. (2011). Toxicology Handbook. Travel Med. Infect. Dis.

Lemire, J., Kumar, P., Mailloux, R., Cossar, K. & Appanna, V.D. (2008). Metabolic adaptation and oxaloacetate homeostasis in P. fluorescens exposed to aluminum toxicity. J. Basic Microbiol., 48, 252–259.

Leung, M.C.K., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., et al. (2008). Caenorhabditis elegans: an emerging model in biomedical and environmental toxicology. Toxicol. Sci., 106, 5–28.

Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J.H., Takeichi, N., et al. (1991). Spontaneous hepatic copper accumulation in long-evans cinnamon rats with hereditary hepatitis: A model of Wilson's disease. J. Clin. Invest., 87, 1858–1861.

Llobet, J.M., Domingo, J.L., Colomina, M.T., Mayayo, E. & Corbella, J. (1988). Subchronic oral toxicity of zinc in rats. Bull. Environ. Contam. Toxicol., 41, 36–43.

M. S. Yang. (1998). Determination of endogenous trace metal contents in various mouse brain regions after prolonged oral administration of aluminum chloride. Toxicol. Environ. Heal., 55, 445–453.

Maazouzi, C., Masson, G., Izquierdo, M.S. & Pihan, J.-C. (2008). Chronic copper exposure and fatty acid composition of the amphipod Dikerogammarus villosus: results from a field study. Environ. Pollut., 156, 221–6.

Macomber, L. & Hausinger, R.P. (2011). Mechanisms of nickel toxicity in microorganisms. Metallomics, 3, 1153–62.

Maret, W. (2013). Zinc and human disease. Met. Ions Life Sci., 13, 389–414.

Martinez-Finley, E.J. & Aschner, M. (2011). Revelations from the Nematode Caenorhabditis elegans on the Complex Interplay of Metal Toxicological Mechanisms. J. Toxicol., 2011, 895236.

Massoud, S.A. & Fahmy, M.A. (1994). Heavy Metal Pollution in Coastal Red Sea Waters, Jeddah. J. KAU. Sci, 7, 67–74.

McNamara, R.A.Y. and P.J. (2001). Aluminium toxicokinetics: an updated minireview. Pharmacol. Toxicol., 88, 159–167.

Middendorf, P.J. & Dusenbery, D.B. (1993). Fluoroacetic Acid Is a Potent and Specific Inhibitor of Reproduction in the Nematode Caenorhabditis elegans. J. Nematol., 25, 573–577.

Miles, a T., Hawksworth, G.M., Beattie, J.H. & Rodilla, V. (2000). Induction, regulation, degradation, and biological significance of mammalian metallothioneins. Crit. Rev. Biochem. Mol. Biol., 35, 35–70.

Miller, A. (2002). Subset selection in regression. 2nd edn. Chapman and Hall/CRC, London.

Mohorjy, A.M. & Khan, A.M. (2006). Preliminary Assessment of Water Quality along the Red Sea Coast near Jeddah, Saudi Arabia. Int. Water Resour. Assoc., 31, 109–115.

Montaser, M., Mahfouz, M.E., El-shazly, S.A.M.S.A., Abdel-Rahman, G.H. & Bakry, S. (2010). Toxicity of Heavy Metals on Fish at Jeddah Coast KSA: Metallothionein Expression as a Biomarker and Histopathological Study on Liver and Gills. World J. Fish Mar. Sci., 2, 174–185.

Mörck, C., Olsen, L., Kurth, C., Persson, A., Storm, N.J., Svensson, E., et al. (2009). Statins inhibit protein lipidation and induce the unfolded protein response in the non-sterol

producing nematode Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A., 106, 18285–18290.

Moriwaki, H., Osborne, M.R. & Phillips, D.H. (2008). Effects of mixing metal ions on oxidative DNA damage mediated by a Fenton-type reduction. Toxicol. In Vitro, 22, 36–44.

Mullaney, B.C. & Ashrafi, K. (2009). C. elegans fat storage and metabolic regulation. Biochim. Biophys. Acta, 1791, 474–478.

Nayak, P. & Chatterjee, a. . (2001). Effects of aluminium exposure on brain glutamate and GABA systems: an experimental study in rats. Food Chem. Toxicol., 39, 1285–1289.

Nelson, N. (1999). Metal ion transporters and homeostasis. Mol. Micrbiology, 28, 403–412.

Nestor, J.J. (2009). The medicinal chemistry of peptides. Curr. Med. Chem., 16, 4399–4418.

Nordberg, M. (1998). Metallothioneins: Historical review and state of knowledge. In: Talanta. pp. 243–254.

Norey, C.G. & Crver, A. (1990). A comparison of cadmium-induced metallothionein gene expression and Me2+ distribution in the tissues of cadmiumsensitive (rainbow trout; Salmo gairdneri) and tolerant (stone loach; Noemacheilus barbatulus) species of freshwater fish. Comp. Biochem. Physiol., 9, 221–225.

Olowu, R.A., Ayejuyo, O.O., Adewuyi, G.O., Adejoro, I.A.B., Denloye, A.A., Babatunde, A.O., et al. (2010). Determination of Heavy Metals in Fish Tissues, Water and Sediment from Epe and Badagry Lagoons, Lagos, Nigeria. E-J Chem, 7, 215–221.

Ondreička, R., Ginter, E. & Kortus, J. (1966). Chronic toxicity of aluminium in rats and mice and its effects on phosphorous metabolism. Br. J. Ind. Med.

Van Der Oost, R., Beyer, J. & Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol., 13, 57–149.

Osredkar, J. (2011). Copper and Zinc, Biological Role and Significance of Copper Zinc Imbalance. J. Clin. Toxicol., S:3.

Page, K.E., White, K.N., McCrohan, C.R., Killilea, D.W. & Lithgow, G.J. (2012). Aluminium exposure disrupts elemental homeostasis in Caenorhabditis elegans. Metallomics, 4, 512.

Pagenkopf, G.K. (1983). Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environ. Sci. Technol., 17, 342–347.

Pelgrom, S.M.G.J., Lamers, L.P.M., Lock, R.A.C., Balm, P.H.M. & Bonga, S.E.W. (1995). Interactions between copper and cadmium modify metal organ distribution in mature tilapia Oreochromis mossambicus. Environ. Pollut., 90, 415–423.

Perez, C.L. & Van Gilst, M.R. (2008). A 13C Isotope Labeling Strategy Reveals the Influence of Insulin Signaling on Lipogenesis in C. elegans. Cell Metab., 8, 266–274.

PERSGA/GEF. (2003). Coral Reefs in the Red Sea and Gulf of Aden Surveys 1990 to 2000 Summary and Recommendations. Jeddah.

Plum, L.M., Rink, L. & Haase, H. (2010). The essential toxin: impact of zinc on human health. Int. J. Environ. Res. Public Health, 7, 1342–1365.

Prato, E., Parlapiano, I. & Biandolino, F. (2013). Sublethal effects of copper on some biological traits of the amphipod Gammarus aequicauda reared under laboratory conditions. Chemosphere, 93, 1015–22.

Prousek, J. (2007). Fenton chemistry in biology and medicine. Pure Appl. Chem., 79, 2325–2338.

Rainbow, P.S. & Luoma, S.N. (2011). Metal toxicity, uptake and bioaccumulation in aquatic invertebrates--modelling zinc in crustaceans. Aquat. Toxicol., 105, 455–65.

Rand, G.M. & Petrocelli, S.R. (1985). Fundamentals of aquatic toxicology: methods and applications. Fundam. Aquat. Toxicol. Hemisphere Publishing.

Rao, A.U., Carta, L.K., Lesuisse, E. & Hamza, I. (2005). Lack of heme synthesis in a free-living eukaryote. Proc. Natl. Acad. Sci. U. S. A., 102, 4270–4275.

Rappleye, C.A., Tagawa, A., Le Bot, N., Ahringer, J. & Aroian, R. V. (2003). Involvement of fatty acid pathways and cortical interaction of the pronuclear complex in Caenorhabditis elegans embryonic polarity. BMC Dev. Biol., 3.

Ratner, B. (2011). Statistical and machine-learning data mining: Techniques for better predictive modeling and analysis of big data. 2nd edn. Taylor and Francis Group, Boca Raton.

Ribeiro, F., Ferreira, N.C.G., Ferreira, A., Soares, A.M.V.M. & Loureiro, S. (2011). Is ultraviolet radiation a synergistic stressor in combined exposures? The case study of Daphnia magna exposure to UV and carbendazim. Aquat. Toxicol., 102, 114–122.

Rikans, L.E. & Hornbrook, K.R. (1997). Lipid peroxidation, antioxidant protection and aging. Biochim. Biophys. Acta, 1362, 116–127.

Riley, J.P. & Segar, D.A. (1970). The distribution of major and some minor elements in marine animals. 1- Echinoderms and Coelenterates. J. Mar. Biol. Assoc. U.K., 50, 721–730.

Rodríguez-Montelongo, L., Volentini, S.I., Farías, R.N., Massa, E.M. & Rapisarda, V. a. (2006). The Cu(II)-reductase NADH dehydrogenase-2 of Escherichia coli improves the bacterial growth in extreme copper concentrations and increases the resistance to the damage caused by copper and hydroperoxide. Arch. Biochem. Biophys., 451, 1–7.

Roesijadi, G. (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat. Toxicol.

Roh, H.C., Collier, S., Guthrie, J., Robertson, J.D. & Kornfeld, K. (2012). Lysosome-related organelles in intestinal cells are a zinc storage site in C. elegans. Cell Metab., 15, 88–99.

Rolfs, A. & Hediger, M. (1999). Metal ion transporters in mammals: structure, function and pathological implications. J. Physiol., 1–12.

Santore, R.C., Mathew, R., Paquin, P.R. & DiToro, D. (2002). Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and Daphnia magna. Comp. Biochem. Physiol. - C Toxicol. Pharmacol.

Shahidul Islam, M. & Tanaka, M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Mar. Pollut. Bull., 48, 624–649.

Shanker, A. (2008). Mode of Action and Toxicity of Trace Elements. Trace Elem. as Contam. Nutr., 523–553.

Shivay, Y. & Kumar, D. (2005). Effect of Phosphorous and Zinc Fertilisation on the Productivity of Transplanted Aromatic Rice. Micronutr. South South East Asia, 199–204.

Smet, H. De, Wachter, B. De, Lobinski, R. & Blust, R. (2001). Dynamics of (Cd, Zn)-metallothioneins in gills, liver and kidney of common carp Cyprinus carpio during cadmium exposure. Aquat. Toxicol., 52, 269–281.

Speisky, H., Gómez, M., Burgos-Bravo, F., López-Alarcón, C., Jullian, C., Olea-Azar, C., et al. (2009). Generation of superoxide radicals by copper-glutathione complexes: redox-consequences associated with their interaction with reduced glutathione. Bioorg. Med. Chem., 17, 1803–1810.

Spry, D. J. and Wood, C. M. (1984). Acid-base, plasma ion and blood-gas changes in rainbow-trout during short-term toxic zinc exposure. *Journal of Comparative Physiology* 154, 149-158.

Spry, D. J. and Wood, C. M. (1985). Ion flux rates, acid-base status, and blood-gases in rainbow-trout, salmo-gairdneri, exposed to toxic zinc in natural soft-water. *Canadian Journal of Fisheries and Aquatic Sciences* 42, 1332-1341.

Spurgeon, D.J., Jones, O. a H., Dorne, J.-L.C.M., Svendsen, C., Swain, S. & Stürzenbaum, S.R. (2010). Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. Sci. Total Environ., 408, 3725–3734.

Stadtman, E. (1990). Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic. Biol. Med., 9, 315–325.

Sugimoto, M., T. Oikawa, N. Esaki, H.T. and K.S. (1988). Chemical synthesis and expression of copper metalothioneien gene of Neurospora crassa. J. Biochem, 104, 924–926.

Tamás Wysocki, R., M.J. (2001). Mechanisms involved in metalloid transport and tolerance acquisition. Curr Genet, 40, 2–12.

Tatara, C. (1998). Use of ion characteristics to predict relative toxicity of mono-, di-and trivalent metal ions: Caenorhabditis elegans LC50. Aquat. Toxicol., 42, 255–269.

Taylor, W.M. & Halperin, M.L. (1975). Effect of glutamate on the control of fatty-acid synthesis in white adipose tissue of the rat. Inhibition of pyruvate dehydrogenase. Eur. J. Biochem., 53, 411–418.

Toro, D. Di & Allen, H. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ., 20, 2383–2396.

Trabulsi, M.Y. (2006). Jeddah: A story of a City. Riyadh: King Fahd Library.

U.S. EPA (Environmental Protection Agency). (2007). Framework for metals risk assessment, EPA 120/R-07/001. U.S. EPA. Washington, DC.

USEPA. (1988). Guidance for Reporting on the Environmental Fate and Transport of the Stressors of Concern in Problem Formulations. Washington, D.C.

USEPA. (1997). Recommended guidelines for measuring metals in puge sound marine water, sediment and tissue samples. Washington, D.C.

Uysal, K., Emre, Yi. & Köse, E. (2008). The determination of heavy metal accumulation ratios in muscle, skin and gills of some migratory fish species by inductively coupled plasma-optical emission spectrometry (ICP-OES) in Beymelek Lagoon (Antalya/Turkey). Microchem J, 90, 67–70.

Vaidyanathan, S. (2005). Profiling microbial metabolomes: what do we stand to gain? Metabolomics.

Valko, M., Morris, H. & Cronin, M.T.D. (2005). Metals, toxicity and oxidative stress. Curr. Med. Chem., 12, 1161–1208.

Viant, M.R., Rosenblum, E.S. & Tjeerdema, R.S. (2003). NMR-Based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health. Environ. Sci. Technol., 37, 4982–4989.

Waldron, K.J., Rutherford, J.C., Ford, D. & Robinson, N.J. (2009). Metalloproteins and metal sensing. Nature, 460, 823–830.

Wang, F., Wang, X., Liu, Y., Tian, W.-X. & Zhou, H.-M. (2003). Inhibitive effect of zinc ion on fatty acid synthase from chicken liver. Int. J. Biochem. Cell Biol., 35, 391–400.

Wapnir, R.A. & Stiel, A. (1988). Zinc Intestinal Absorption Amino Acids as Ligands in Rats : Specificity of, 2171–2179.

Wastney, M., Aamodt, R., Rumble, W. & Henkin, R. (1986). Kinetic analysis of zinc metabolism and its regulation in normal humans. Am. J. Physiol. - Regul. Integr. Comp. Physiol., 251, 398–408.

Watts, D. (1989). The nutritional relationships of copper. Orthomol. Med., 4, 99–108.

Watts, J.L. (2009). Fat synthesis and adiposity regulation in Caenorhabditis elegans. Trends Endocrinol. Metab., 20, 58–65.

Watts, M.J., Barlow, T.S., Button, M., Sarkar, S.K., Bhattacharya, B.D., Alam, M.A., et al. (2013). Arsenic speciation in polychaetes (Annelida) and sediments from the intertidal mudflat of Sundarban mangrove wetland, India. Environ. Geochem. Health, 35, 13–25.

Whm, V.K., Rwb, Z. & W, S. (1989). Testing environmental pollutants on soil organisms: A simple assay to investigate toxicity on soil organisms, using CdCl2 and nematodes. Ecotoxicol. Environ. Saf., 18, 181–190.

WHO. (1996). Trace elements in human nutrition and health. Med. Clin. North Am.

Williams, P.L. (1990). Aquatic toxicity testing using the nematode, Caenorhabditis elegans. Environ. Toxicol., 9, 1285–1290.

Williams, P.L. & Dusenbery, D.B. (1988). Using the nematode Caenorhabditis elegans to predict mammalian acute lethality to metallic salts. Toxicol. Ind. Health, 4, 469–478.

Williams, R.J. (1999). What is wrong with aluminium? The J.D. Birchall Memorial Lecture. J. Inorg. Biochem., 76, 81–88.

Wu, J., Forbes, J.R., Chen, H.S. & Cox, D.W. (1994). The LEC rat has a deletion in the copper transporting ATPase gene homologous to the Wilson disease gene. Nat. Genet., 7, 541–545.

Yamasaki, S., Sakata-Sogawa, K., Hasegawa, A., Suzuki, T., Kabu, K., Sato, E., et al. (2007). Zinc is a novel intracellular second messenger. J. Cell Biol., 177, 637–645.

YAN, X. (2009). Linear regression analysis: theory and computing. World Scientific, Singapore.

Yang, H. (2013). The case for being automatic: Introducing the automatic linear modelling (LINEAR) procedure in SPSS statistics. Mult. Linear Regres. Viewpoints, 39, 27–37.

Yang, M.S., Wong, H.F., Yung, K.L. & M. S. Yang. (1998). Determination of endogenous trace metal contents in various mouse brain regions after prolonged oral administration of aluminum chloride. Toxicol. Environ. Heal., 55, 445–453.

Yoon, S., Han, S.S. & Rana, S. (2008). Molecular markers of heavy metal toxicity- A new paradigm for health risk assessment. J. Environ. Biol., 29, 1–14.

Zaman, K., Zaman, W. & Siddique, H. (1993). Hematological and enzymatic results of aluminum intoxication in rats. Comp. Biochem. Physiol. C., 105, 73–76.

Zhang, L., Liu, X., You, L., Zhou, D., Wu, H., Li, L., et al. (2011). Metabolic responses in gills of Manila clam Ruditapes philippinarum exposed to copper using NMR-based metabolomics. Mar. Environ. Res., 72, 33–9.

Zitko, V. & Carson, W. (1976). A mechanism of the effects of water hardness on the lethality of heavy metals to fish. Chemosphere, 1–5.

12 APPENDIX

Table 17: Changes in composition of elements present in control, and Cu doses exposed to nematodes. (19 µM, 37 µM, 56 µM and 75 µM) as measured by ICP-MS. The average µg of element/mg of dry nematode sample mass (Ave), and SDEV is shown for each concentration. ***P*** Values are shown for each value compared to the control via t-test, **ns** represents no significance ($p < 0.05$), and ***** represents significant difference ($p < 0.05$).

	Control		**Cu 19 µM**			**Cu 37 µM**			**Cu 56 µM**			**Cu 75 µM**		
Element	Ave	SDEV	Ave	SDEV	***P*** value/	Ave	SDEV	***P*** value/	Ave	SDEV	***P*** value/	Ave	SDEV	***P*** value/
Li	0.15	0.10	0.02	0.02	*	0.11	0.07	ns	0.158	0.11	ns	0.072	0.06	ns
Be	0.01	0.02	0.00	0.01	ns	0.00	0.00	ns	0.000	0.00	ns	0.000	0.00	ns
Mg	75.82	21.92	45.77	11.15	*	50.60	3.85	*	50.250	6.63	*	35.125	11.40	*
Al	116.61	57.77	110.60	14.11	ns	105.40	34.17	ns	145.889	22.11	ns	165.380	63.21	ns
P	836.06	262.06	538.41	138.33	ns	650.56	58.13	ns	628.631	70.42	ns	457.390	161.78	*
S	346.93	109.04	286.26	55.91	ns	329.99	69.08	ns	316.575	65.58	ns	196.001	101.64	ns
Ca	67.26	22.99	56.09	6.03	ns	52.62	5.21	ns	72.536	16.03	ns	65.349	16.87	ns
Ti	689.12	440.81	348.73	76.30	ns	377.59	136.25	ns	481.758	207.66	ns	594.782	283.39	ns
V	0.10	0.09	0.01	0.00	ns	0.02	0.00	ns	0.013	0.01	ns	0.007	0.01	ns
Cr	0.18	0.13	0.12	0.03	ns	0.09	0.06	ns	0.208	0.05	ns	0.237	0.16	ns
Mn	1.05	0.42	0.52	0.08	*	0.58	0.05	*	0.458	0.05	*	0.324	0.12	*
Fe	11.81	5.81	6.86	1.16	ns	7.55	2.31	ns	9.494	3.97	ns	7.004	1.86	ns
Co	0.01	0.01	0.01	0.00	ns	0.01	0.00	ns	0.007	0.00	ns	0.006	0.00	ns
Ni	0.09	0.04	0.06	0.02	ns	0.09	0.10	ns	0.095	0.02	ns	0.098	0.05	ns
Cu	0.32	0.11	2.65	0.67	*	11.96	4.39	*	17.769	7.96	*	22.788	8.28	*
Zn	3.62	2.57	1.85	0.61	ns	2.71	0.57	ns	2.336	0.55	ns	1.453	0.88	ns
Ga	0.03	0.01	0.03	0.01	ns	0.02	0.01	ns	0.031	0.01	ns	0.037	0.02	ns
As	2.81	1.09	2.29	0.23	ns	2.47	0.59	ns	2.709	0.28	ns	3.190	1.02	ns
Se	0.04	0.02	0.02	0.00	*	0.03	0.01	ns	0.021	0.00	*	0.014	0.01	*

Continued Table 17: Changes in composition of elements present in control, and Cu doses exposed to nematodes. (19 µM, 37 µM, 56 µM and 75 µM) as measured by ICP-MS. The average µg of element/mg of dry nematode sample mass (Ave), and SDEV is shown for each concentration. ***P*** Values are shown for each value compared to the control via t-test, **ns** represents no significance ($p < 0.05$), and ***** represents significant difference ($p < 0.05$).

	Control		**Cu 19 µM**			**Cu 37 µM**			**Cu 56 µM**			**Cu 75 µM**		
Element	Ave	SDEV	Ave	SDEV	***P*** value/	Ave	SDEV	***P*** value/	Ave	SDEV	***P*** value/	Ave	SDEV	***P*** value/
Rb	0.08	0.04	0.05	0.01	ns	0.04	0.01	ns	0.050	0.00	ns	0.045	0.01	ns
Sr	0.20	0.08	0.15	0.02	ns	0.13	0.01	ns	0.169	0.02	ns	0.168	0.05	ns
Y	0.02	0.01	0.02	0.00	ns	0.02	0.00	ns	0.023	0.01	ns	0.023	0.01	ns
Zr	0.13	0.19	0.00	0.00	ns	0.08	0.07	ns	0.064	0.07	ns	0.200	0.26	ns
Nb	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.002	0.00	ns	0.001	0.00	ns
Mo	0.08	0.05	0.02	0.00	ns	0.05	0.01	ns	0.044	0.01	ns	0.033	0.01	ns
Ag	0.03	0.02	0.01	0.00	*	0.02	0.01	ns	0.015	0.01	ns	0.010	0.01	ns
Cd	0.04	0.02	0.02	0.00	*	0.07	0.10	ns	0.017	0.00	*	0.014	0.01	*
Sn	0.01	0.01	0.02	0.01	ns	0.07	0.14	ns	0.008	0.02	ns	0.048	0.03	*
Sb	0.01	0.00	0.00	0.00	ns	0.00	0.00	ns	0.003	0.00	ns	0.003	0.00	ns
Cs	0.05	0.03	0.01	0.01	*	0.01	0.00	*	0.010	0.01	*	0.008	0.01	*
Ba	3.54	1.16	3.44	0.34	ns	3.19	0.77	ns	4.046	0.44	ns	4.725	1.57	ns
La	0.02	0.01	0.02	0.00	ns	0.02	0.00	ns	0.027	0.00	ns	0.065	0.08	ns
Ce	0.09	0.04	0.10	0.01	ns	0.10	0.02	ns	0.115	0.01	ns	0.148	0.05	ns
Pr	0.01	0.00	0.01	0.00	ns	0.00	0.00	ns	0.006	0.00	ns	0.007	0.00	ns
Nd	0.02	0.01	0.02	0.00	ns	0.02	0.01	ns	0.022	0.01	ns	0.026	0.01	ns
Sm	0.00	0.00	0.01	0.00	ns	0.00	0.00	ns	0.004	0.00	ns	0.006	0.00	ns
Eu	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.001	0.00	ns	0.001	0.00	ns
Tb	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.001	0.00	ns	0.001	0.00	ns

Continued Table 17: Changes in composition of elements presented in control and Cu doses exposed to nematodes. (19 µM, 37 µM, 56 µM and 75 µM) as measured by ICP-MS. The average µg of element/mg of dry nematode sample mass (Ave), and SDEV is shown for each concentration. *P* values are shown for each value compared to the control via t-test, ns represented no significant (p <0.05) and * represents significant difference ($p < 0.05$).

	Control		**Cu 19 µM**			**Cu 37 µM**			**Cu 56 µM**			**Cu 75 µM**		
Element	Ave	SDEV	Ave	SDEV	***P*** value/	Element	Ave	SDEV	Ave	SDEV	***P*** value/	Element	Ave	SDEV
Gd	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.004	0.00	ns	0.004	0.00	ns
Dy	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.005	0.00	ns	0.003	0.00	ns
Ho	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.001	0.00	ns	0.001	0.00	ns
Er	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.002	0.00	ns	0.002	0.00	ns
Tm	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.000	0.00	ns	0.000	0.00	ns
Yb	0.00	0.00	0.00	0.00	ns	0.00	0.00	*	0.002	0.00	ns	0.002	0.00	ns
Lu	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.000	0.00	ns	0.000	0.00	*
W	0.01	0.01	0.00	0.00	ns	0.00	0.00	ns	0.003	0.00	ns	0.003	0.00	ns
Tl	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.000	0.00	ns	0.000	0.00	ns
Pb	0.11	0.04	0.11	0.01	ns	0.17	0.11	ns	0.139	0.01	ns	0.157	0.04	ns
Bi	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.004	0.00	ns	0.002	0.00	ns
Th	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.004	0.00	ns	0.005	0.00	ns
U	0.01	0.00	0.02	0.02	ns	0.00	0.00	ns	0.158	0.11	ns	0.072	0.06	ns

Table 18: Changes in composition of elements present in control, and Zn doses exposed to nematodes. (340 µM, 679 µM, 1019 µM and 1358 µM) as measured by ICP-MS. The average µg of element/mg of dry nematode sample mass (Ave), and SDEV is shown for each concentration. ***P*** Values are shown for each value compared to the control via t-test, **ns** represents no significance ($p < 0.05$), and ***** represents significant difference ($p < 0.05$).

	Control		**Zn 340 µM**			**Zn 679 µM**			**Zn 1019 µM**			**Zn 1358 µM**		
Elemen	Ave	SDEV	Ave	SDEV	***P*** value	Ave	SDEV	***P*** value	Ave	SDEV	***P*** value	Ave	SDEV	***P*** value
Li	0.15	0.10	0.13	0.17	ns	0.04	0.01	ns	0.02	0.03	*	0.02	0.04	*
Be	0.01	0.02	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
Mg	75.82	21.92	45.39	8.39	*	41.82	3.66	*	42.38	5.99	*	51.83	9.54	ns
Al	116.61	57.77	144	116	ns	90.04	13.59	ns	66.12	19.20	ns	41.04	10.36	*
P	836	262	743	178	ns	969	61.76	ns	1144	138	*	1494	224	*
S	346	109	322	161	ns	209	47.48	*	189	38.20	*	198	45.38	*
Ca	67.26	22.99	66.57	25.74	ns	49.48	9.72	ns	54.23	10.75	ns	40.71	10.33	*
Ti	689	440	745	590	ns	328	71.06	ns	220	168	ns	135	72.49	*
V	0.10	0.09	0.02	0.01	ns	0.02	0.01	ns	0.02	0.01	ns	0.03	0.01	ns
Cr	0.18	0.13	0.22	0.24	ns	0.08	0.08	ns	0.02	0.03	*	0.00	0.00	*
Mn	1.05	0.42	0.50	0.07	*	0.39	0.08	*	0.28	0.06	*	0.27	0.03	*
Fe	11.81	5.81	8.61	3.91	ns	5.51	0.55	*	4.53	0.73	*	3.35	0.36	*
Co	0.01	0.01	0.01	0.00	ns	0.01	0.00	ns	0.00	0.00	ns	0.01	0.00	ns
Ni	0.09	0.04	0.10	0.10	ns	0.04	0.03	*	0.02	0.01	*	0.00	0.00	*
Cu	0.32	0.11	0.39	0.13	ns	0.34	0.07	ns	0.39	0.05	ns	0.32	0.04	ns
Ga	0.03	0.01	0.03	0.02	ns	0.02	0.01	ns	0.02	0.01	*	0.01	0.00	*
As	2.81	1.09	2.81	1.97	ns	1.99	0.29	ns	1.54	0.33	*	1.17	0.14	*
Se	0.04	0.02	0.02	0.01	*	0.02	0.00	*	0.02	0.00	*	0.02	0.00	*
Rb	0.08	0.04	0.04	0.02	ns	0.04	0.00	ns	0.04	0.00	ns	0.04	0.01	ns
Sr	0.20	0.08	0.21	0.12	ns	0.14	0.01	ns	0.11	0.01	*	0.10	0.05	*

Continued Table 18: Changes in composition of elements present in control, and Zn doses exposed to nematodes. (340 µM, 679 µM, 1019 µM and 1358 µM) as measured by ICP-MS. The average µg of element/mg of dry nematode sample mass (Ave), and SD is shown for each concentration. *P* Values are shown for each value compared to the control via t-test, ns represents no significance ($p < 0.05$), and * represents significant difference ($p < 0.05$).

	Control		**Zn 340 µM**			**Zn 679 µM**			**Zn 1019 µM**			**Zn 1358 µM**		
Element	Ave	SD	Ave	SD	***P*** value/	Ave	SD	***P*** value/	Ave	SDV	***P*** value/	Ave	SD	***P*** value/
Sr	0.20	0.08	0.21	0.12	ns	0.14	0.01	ns	0.11	0.01	*	0.10	0.05	*
Y	0.02	0.01	0.02	0.02	ns	0.02	0.00	ns	0.01	0.00	ns	0.01	0.00	*
Zr	0.13	0.19	0.35	0.49	ns	0.01	0.03	ns	0.03	0.04	ns	0.00	0.01	ns
Nb	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*
Mo	0.08	0.05	0.05	0.02	ns	0.02	0.01	ns	0.02	0.01	*	0.02	0.01	*
Ag	0.03	0.02	0.11	0.01	*	0.31	0.10	*	0.53	0.15	*	0.57	0.14	*
Cd	0.04	0.02	0.01	0.00	*	0.01	0.00	*	0.01	0.00	*	0.01	0.00	*
Sn	0.01	0.01	0.03	0.02	ns	0.01	0.01	ns	0.01	0.01	ns	0.00	0.01	ns
Sb	0.01	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
Cs	0.05	0.03	0.01	0.01	*	0.00	0.00	*	0.00	0.00	*	0.00	0.00	*
Ba	3.54	1.16	4.14	2.83	ns	2.86	0.36	ns	2.18	0.50	*	1.64	0.22	*
La	0.02	0.01	0.03	0.02	ns	0.02	0.00	ns	0.05	0.08	ns	0.01	0.00	*
Ce	0.09	0.04	0.13	0.08	ns	0.11	0.01	ns	0.14	0.14	ns	0.07	0.01	ns
Pr	0.01	0.00	0.01	0.00	ns	0.00	0.00	ns	0.01	0.01	ns	0.00	0.00	*
Nd	0.02	0.01	0.03	0.02	ns	0.02	0.00	ns	0.02	0.02	ns	0.01	0.00	ns
Sm	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*
Eu	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
Tb	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*
Gd	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*
Dy	0.00	0.00	0.01	0.00	ns	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*

Continued Table 18: Changes in composition of elements present in control, and Zn doses exposed to nematodes. (340 µM, 679 µM, 1019 µM and 1358 µM) as measured by ICP-MS. The average µg of element/mg of dry nematode sample mass (Ave), and SDEV is shown for each concentration. *P* Values are shown for each value compared to the control via t-test, ns represents no significance ($p < 0.05$), and * represents significant difference ($p < 0.05$).

	Control		**Zn 340 µM**			**Zn 679 µM**			**Zn 1019 µM**			**Zn 1358 µM**		
Element	Ave	SDE	Ave	SDE	***P*** value	Ave	SDE	***P*** value	Ave	SDEV	***P*** value	Ave	SDE	***P*** value
Ho	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
Er	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*
Tm	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*
Yb	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*
Lu	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*
W	0.01	0.01	0.01	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
Tl	0.00	0.00	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
Pb	0.11	0.04	0.15	0.09	ns	0.12	0.01	ns	0.12	0.04	ns	0.09	0.01	ns
Bi	0.00	0.00	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*	0.00	0.00	*
Th	0.00	0.00	0.01	0.00	ns	0.00	0.00	ns	0.00	0.00	ns	0.00	0.00	ns
U	0.01	0.00	0.01	0.00	ns	0.00	0.00	ns	0.00	0.00	*	0.00	0.00	*

Table 19: A list of identified trace metals compositions measured by ICP−MS Data. After ANOVA test has made and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ are represented the mean difference of the exposed samples compared to the mean of the control sample.

Elements	Cu LC_{10}	Cu LC_{20}	Cu LC_{30}	Cu LC_{40}	Zn LC_{10}	Zn LC_{20}	Zn LC_{30}	Zn LC_{40}	Al LC_{10}	Al LC_{20}	Al LC_{30}	Al LC_{40}
Ag	↓	↓			↑	↑	↑	↑	↓			
Al								↓		↑	↑	↑
As							↓	↓		↓	↓	↓
Ba							↓	↓		↓	↓	↓
Be									↓			
Bi		↑				↓	↓	↓	↓		↓	↓
Ca								↓			↓	
Cd	↓		↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Ce					↓							
Co									↓			
Cr							↓	↓		↓	↓	↓
Cs	↓	↓	↓	↓		↓	↓	↓	↓	↓	↓	↓
Dy		↑					↓	↓	↓	↓	↓	↓
Er		↑					↓	↓	↓		↓	↓
Eu		↑							↓			
Fe						↓	↓	↓		↓	↓	↓
Ga		↓					↓	↓	↓			
Gd		↑						↓	↓		↓	↓
Ho		↑							↓			
La		↑						↓	↓		↓	↓

Continued Table 19: A list of identified trace metals compositions measured by ICP−MS. Data after ANOVA test has made and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ are represented the mean difference of the exposed samples compared to the mean of the control sample.

Elements	Cu LC_{10}	Cu LC_{20}	Cu LC_{30}	Cu LC_{40}	Zn LC_{10}	Zn LC_{20}	Zn LC_{30}	Zn LC_{40}	Al LC_{10}	Al LC_{20}	Al LC_{30}	Al LC_{40}
Li	↑						↓	↓		↓	↓	↓
Lu		↑		↑			↓	↓	↓		↓	↓
Mg	↓		↓	↓	↓	↓	↓				↓	
Mn	↓		↓	↓	↓	↓	↓	↓		↓	↓	↓
Mo							↓	↓	↓		↓	↓
Nb		↑						↓	↓			
Nd		↑							↓			
Ni						↓	↑	↓	↓	↓	↓	↓
P				↓			↓	↑		↑		
Pb		↓										
Pr		↓						↓	↓		↓	

Continued Table 19: A list of identified trace metals compositions measured by ICP−MS. Data after ANOVA test has made and compared to the control via t-test ($p < 0.05$) for significance. ↑↓ are represented the mean difference of the exposed samples compared to the mean of the control sample.

Elements	Cu LC_{10}	Cu LC_{20}	Cu LC_{30}	Cu LC_{40}	Zn LC_{10}	Zn LC_{20}	Zn LC_{30}	Zn LC_{40}	Al LC_{10}	Al LC_{20}	Al LC_{30}	Al LC_{40}
Rb		↓							↓			
S						↓	↓	↓		↓	↓	↓
Sb		↓							↓			
Se	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Sm		↑						↓	↓			
Sn				↑					↓			
Sr							↓	↓			↓	↓
Tb		↑					↓	↓	↓		↓	↓
Th		↑							↓			
Ti								↓			↓	↓
Tl		↑							↓			
Tm		↑						↓	↓		↓	↓
U		↓					↓	↓	↓		↓	↓
V		↓							↓			
W		↓							↓			↓
Y		↑						↓	↓	↓	↓	↓
Yb		↑					↑	↓	↓	↓	↓	↓