UNIVERSITY OF BIRMINGHAM

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A Study Assessing the Comparative Molecular Toxicities of Heavy Metals to Multiple Strains of *Daphnia magna*

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Abstract

The freshwater crustacean *Daphnia magna* is used extensively in ecotoxicological testing throughout the world. In addition, acute toxicity tests using *Daphnia* are required during chemical risk assessment including under EU Registration, Evaluation, Authorisation and restriction of Chemicals (REACH). Multiple studies have reported variation in the response of different strains of *Daphnia* to toxicant exposure including heavy metals.

In this thesis, 10 strains of *Daphnia magna* were obtained from international research institutions and the comparative molecular toxicities of the heavy metals cadmium and copper to these strains was investigated. A standard measure of acute toxicity, the acute immobilisation test was performed, in addition to the use of mass spectrometry-based metabolomics in order to assess sensitivity and metabolic differences between these strains. Up to 12-fold differences in sensitivity between strains of *Daphnia* were observed. Considerable differences in the basal metabolism of these strains was also discovered and correlated with relative sensitivity of the strains to cadmium and copper.

Together these findings show for the first time that strains of *Daphnia magna* are metabolically different in addition to identifying a significant limitation in the use of *Daphnia* in regulatory decision making: results from independent laboratories using different strains of *Daphnia* may not agree.

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List of Abbreviations

4-HNE	4-Hydoxynonenal
BBM	Bolds basal medium
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Cd	Cadmium
Cu	Copper
DCA	3,4-dichloroanaline
DCF-DA	2’7’-dichlorofluorescin diacetate
DFF-DA	2’7’-difluorofluorescin diacetate
DIMS	Direct infusion mass spectrometry
DMSO	Dimethyl sulfoxide
DNTB	5,5’-dithio-bis-2-(nitrobenzoic acid)
EC50	50% of effect concentration
ESI	Electrospray ionisation
EU	European Union
FDR	False discovery rate
FT-ICR-MS	Fourier-transform ion cyclotron mass spectrometry
g-log	Generalised-log
GC-MS	Gas chromatography mass spectrometry
GSH	Glutathione
GSSG	Glutathione disulphide
KNN	K-nearest neighbour
LC (MS)	Liquid chromatography (mass spectrometry)
LC50	50% of lethal concentration
LV	Latent variable
MDA	Malondialdehyde
MS	Mass spectrometry
NMR	Muclear magnetic resonance
NTB	5-thio-2-nitrobenzoic acid
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate buffered saline
PC	Principle component
PCA	Principle component analysis
PLS-DA	Partial least squares discriminant analysis
PQN	probalistic quotient normalisation
QC	Quality control
RAPD	Random amplification of polymorphic DNA
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
ROS	Reactive oxygen species
SIM	Selected ion monitoring
SNR	Signal to noise ratio

SOD	Super oxide dismutase
TSK	Trimmed Sperman-Karber
MES	2-(N-morpholino)ethanesulphonic acid
2-VP	2-vinylpyridine

Chapter 1

Introduction

1.1 Principles of ecotoxicology

Historically toxicological risk assessment has focussed on human exposure, however, it is now recognised that protection of the natural environment is equally important when evaluating the impact of a given substance. The field of ecotoxicology “aims to understand and predict the adverse effects of chemicals on natural communities” (Chapman 2002). In turn, the information gathered during ecotoxicological testing is used to inform on ecosystem protection and regulatory decision making (Breitholtz et al. 2006).

Under current basic test principles information gained from controlled laboratory experiments using individuals from a selected indicator species is used to inform of population level effects (Breitholtz et al. 2006; Chapman 2002). Often, such experiments are designed around four key features; the monetary and social cost including the sacrifice of animals involved, the ecological relevance of the study, in addition to the reliability and sensitivity of the study as a whole (Breitholtz et al. 2006). Standardised tests using only one species generally have a few parameters that may easily be controlled increasing the reproducibility of such results. The relevance of such tests, however is relatively low as they fail to represent effects at other trophic levels in addition to other complex interactions that occur in “real” situations (Breitholtz et al. 2006; Chapman 2002). With an increase in complexity and thus relevance that would come from the introduction of more parameters (e.g. multiple test species), there is a concomitant decrease in reproducibility. The challenge of ecotoxicology remains that within the natural environment there are innumerable combinations of populations and species all participating in complex interactions that simply cannot be recreated within a laboratory setting.

Current standardised ecotoxicological tests assess toxicant effects at a whole organism end point. Acute toxicity tests focus on lethality, including the assessment of 50% lethal concentration (LC_{50}) or other endpoints such as 'immobilisation' where lethality cannot accurately be assessed e.g. through application of the acute immobilisation test (OECD 2004). The effects of chronic toxicant exposure are often assessed through the impact of toxicants on the reproduction of model organisms (OECD 1998). Where lethality cannot be confirmed or other endpoints are measured a 50% effective concentration (EC_{50}) is reported. The significant limitation of such tests is that little in terms of a mechanistic explanation for the results observed can be determined beyond identifying threshold levels of toxicity. In addition, the exposure concentrations used during these studies are often unrealistically high in comparison to environmental levels of compounds of interest. Currently, molecular assays into the underlying mechanisms for toxicity are not required for regulatory decision making (Lilienblum *et al.* 2008).Therefore, information regarding the effects and potential mechanisms of toxicity at realistic exposure levels for environmental contaminants is relatively sparse.

1.2 EU chemical legislation (REACH)

During 2007, new EU (European Union) chemical legislation: REACH (Registration, Evaluation, Authorisation and restriction of CHemicals) came into effect. The main aims of REACH are the wide-scale reorganisation of chemical legislation within the EU with large emphasis on the protection of human health and the environment (Foth and Hayes 2008). It is recognised that in order for the latter to be achieved, the significant gaps in safety assessment data for industrial chemicals need to be addressed (Lilienblum et al. 2008). For the majority of chemicals (produced or imported in volumes in excess of 1 tonne/year) brought onto the market between 1971-1981, of which there are approximately 100,000, there is little or no safety assessment or toxicological data (Lilienblum et al. 2008). In

addition, under EU Directive 67/548/EEC all new substances registered must have undergone a full safety assessment including the generation of full toxicological (irritation, sensitisation, mutagenicity etc.) and ecotoxicological profiles (Foth and Hayes 2008; Lilienblum et al. 2008). Ecotoxicological profiles required under REACH legislation include; growth inhibition studies using algae and acute and chronic toxicity studies in *Daphnia magna* and fish, in order to investigate effect of potential toxicants at several trophic levels within aquatic systems (Foth and Hayes 2008). The number of animals needed to fulfil these demands for all the necessary chemicals has been estimated to be anywhere between 3 and 45 million (Hofer et al. 2004; Van der Jagt et al. 2004). However, one of the main aims of REACH legislation is a reduction in the number of vertebrate tests performed within the EU, with significant emphasis on the 3 R's (Refine, Reduce and Replace). The 3 R's is a commitment to reducing the number of animal experiments performed in life sciences, including reducing the use of vertebrates and/or mammalian species (Lilienblum *et al.* 2008; Russell and Burch 1959). Hence, the information gained at simple earlier tests using *Daphnia magna* and algae is used to inform on the necessity of tests carried out using vertebrate species (Breitholtz et al 2006). In this manner, if no significant toxicant effects are observed in invertebrate species the level and extent of testing conducted in vertebrates is reduced accordingly.

1.3 *Daphnia magna* in ecotoxicology

Amongst the ecotoxicological data required for ecological risk assessment including REACH are acute toxicity experiments using *Daphnia magna* (Tatarazako and Oda 2007). Daphnia are also a test species recommended by the Organisation for Economic Cooperation and Development (OECD), an organisation committed to providing independent guidelines on toxicity testing amongst other social and economic issues (OECD 2002).

Daphnia, more commonly referred to as “water fleas”, are small crustaceans of the order Cladocera (figure 1.1) (Tatarazako and Oda 2007). Their habitat encompasses fresh water ecosystems world-wide (Tsui and Wang 2007; Ślusarczyk and Pietrzak 2008). In times of abundant resources, *Daphnia* reproduce asexually by means of a process known as parthenogenesis (meaning “virgin birth”), which facilitates rapid clonal expansion. *Daphnia* are also known to switch reproductive strategy to sexual reproduction under stress, for example in response to environmental stimuli such as a shortening photoperiod (Oda et al. 2006). The reproduction of Daphnia is also known to be affected by toxicants, and as such is routinely used to assess the potential toxicity of many compounds (Kooijman and Bedaux 1996).

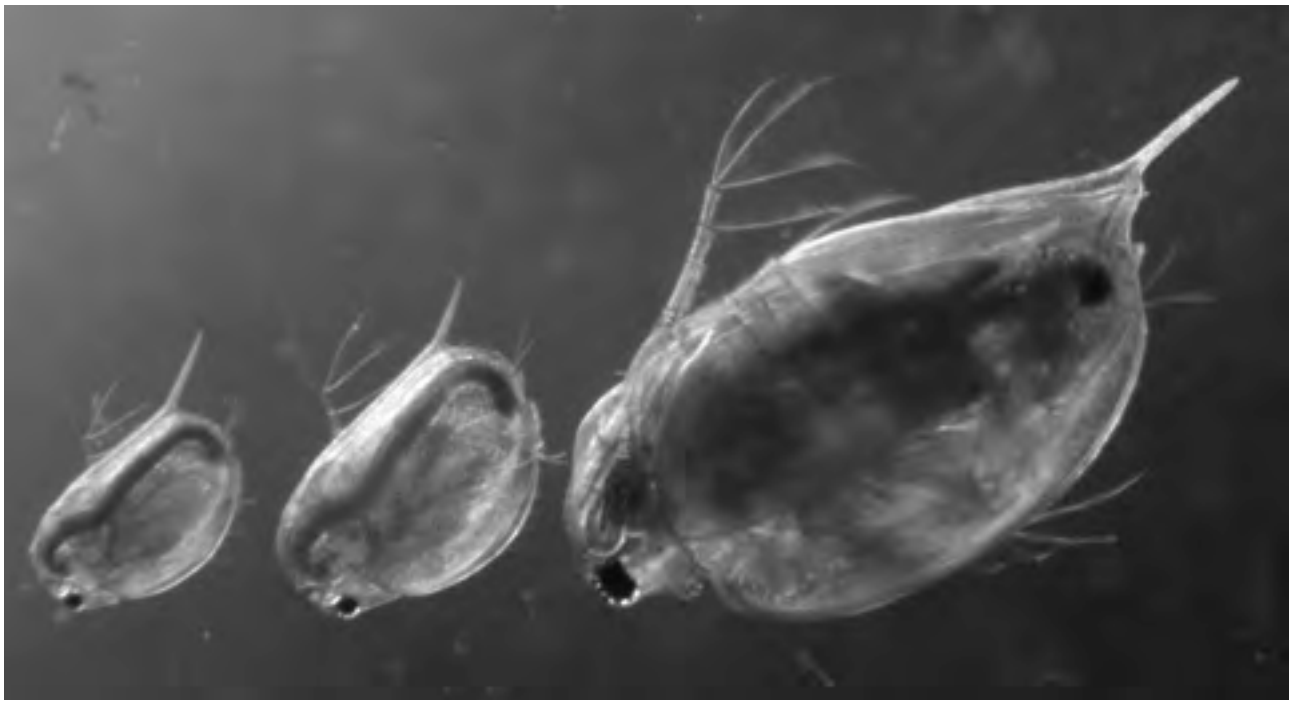


Figure 1.1 Image of *Daphnia magna* (University of Birmingham culture) of various ages including neonate (left) and adult (right)

Daphnia are an attractive test species for ecotoxicological risk assessment for a number of reasons. *Daphnia*, along with other zooplankton occupy an important trophic level within the aquatic food web as primary consumers, and are a so called “keystone species” (Tatarazako and Oda 2007; Tsui and Wang 2007). In addition to being of

ecological importance, Daphnia are relatively easy to maintain in a laboratory, being small and prolific with a short life cycle, making them cost effective. Their unusual reproductive strategy also increases reproducibility through a reduction in biological variability (Haap and Köhler 2009). As an invertebrate, the use of Daphnia in place of other aquatic species such as fish, also complies with the 3R's.

Extensive testing using Daphnia magna has already been conducted, as such they are known to be comparatively sensitive to many compounds including heavy metals, and thus are ideally suited as a standard test species (Tatarazako and Oda 2007; Tsui and Wang 2007). Based on all of these qualities, *Daphnia magna* has been chosen as the test organism for this project. Heavy metal toxicity has been extensively investigated in ecotoxicological testing including numerous studies using *Daphnia magna* (Barata *et al.* 2005; Ward and Robinson 2005; Xie *et al.*2006). The heavy metals cadmium and copper were chosen as model toxicants for this thesis based on the mechanism of toxicity of these metals being relatively well understood (Oliveira-Filho et al. 2004, Manzyl et al. 2004; Soetaert *et al.* 2007).

1.4 Copper toxicity

The heavy metal, copper is an abundant naturally occurring metal; it is also present as an environmental contaminant due to its industrial use, for example in the manufacture of electrical equipment and through mining activities (Oliveira-Filho *et al.* 2004). Copper-based compounds are also widely used as fertilisers, fungicides and aquatic herbicides (Mastin and Rodges 2000; Oliveira-Filho *et al.* 2004).

Copper is an essential element, primarily due to its endogenous function as a co-factor for metalloenzymes, it is thus important in many cellular processes such respiration and neurotransmitter function (Oliveira-Filho *et al.* 2004). Metalloenzymes that incorporate copper include super oxide dismutase (SOD) and cytochrome oxidase (Letelier *et al.* 2005;

Knauert and Knauer 2008). Such enzymes exploit the high redox activity of copper to facilitate electron transfer reactions (Letelier *et al.* 2005; Xie *et al.* 2006).

The same characteristic of copper that makes it an essential element i.e. its redox potential, is also the main source of its toxicity (Xie *et al.* 2006). At high levels, copper homeostasis mechanisms including metal binding proteins and chaperones become overwhelmed and copper becomes toxic (Oliveira-Filho *et al.* 2004, Manzyl *et al.* 2004). In humans an excess in copper body burden, most commonly following poisoning, is associated with severe hepatic necrosis and neurological impairment (López de Romaña *et al.* 2011).

The mechanisms of copper toxicity are relatively well understood and centre around its redox capabilities. Copper may exist in a number of oxidation states, it is understood that in the presence of biological reducing power copper ions may participate in the following Harber-Weiss reaction to produce reactive oxygen species (ROS) (Barata *et al.* 2005; Stohs and Bagchi 1995):

$$Cu(II) + O_2^{\bullet -} \text{ -----> } Cu(I) + O_2$$

$$Cu(I) + H_2O_2 \text{ -----> } Cu(II) + {}^{\bullet}OH + OH^-$$

ROS are highly reactive, partially reduced forms of oxygen and encompass a variety of different molecules including; the super oxide $O_2^{\bullet -}$, hydroxyl radical $^{\bullet}OH$, and hydroxyl anion OH^- (Apel and Hirt 2004). ROS are produced by a variety of endogenous sources including oxidative phosphorylation (Livingstone 2001).

An increase in intracellular ROS levels is associated with oxidative stress, where endogenous protective mechanisms such as anti-oxidant enzymes and scavenger molecules become overwhelmed (Livingstone 2001). As a result these highly reactive molecules go on to react with cellular macromolecules. Target molecules include DNA; where base adducts, single stranded breaks and base oxidation occurs, potentially resulting in acquisition of harmful mutations and apoptosis (Kehrer 2000). Unsaturated

lipids within biological membranes including the plasma membrane are also a target for ROS. The ROS molecules abstract hydrogen atoms, resulting in reactive lipid peroxides that go on to propagate further oxidative damage that may lead to the destruction of cellular membranes and cytotoxicity (Kehrer 2000).

1.5 Cadmium toxicity

The heavy metal, cadmium is present as an environmental contaminant in water, sediment and the atmosphere. Sources include contaminated industrial effluents arising from the use of cadmium in the manufacture of batteries pigments and plastics (Nzengue et al 2011; Matović et al 2011). Thus far, an endogenous role for cadmium in the human body has yet to be established, as such it is considered inherently toxic and its use is restricted in many European countries (Nzengue et al 2011).

Possible mechanisms for the toxic effects of cadmium include; interaction with other metals, reaction with protein sulfhydryl groups, effects on DNA structure and gene expression in addition to affecting cellular membranes and ATP production (Matović et al 2011). At a cellular level, studies have reported the production of ROS upon cadmium exposure in addition to associated oxidative stress and lipid peroxidation (Barata et al. 2005)

In addition to human health the presence of high levels of cadmium within the environment has significant effects for aquatic and other terrestrial organisms. Cadmium is known to persist within the environment, with a half-life of 15-20 years, bioaccumulation within tissues increases the exposure risk at higher levels of the food chain (Matović et al. 2011; Cuypers et al. 2010). The effects of cadmium exposure to *Daphnia magna* has been extensively investigated due to its status as a highly toxic environmental contaminant. Despite the extensive work carried out with this compound the mechanism of action has yet to be completely elucidated (Soetaert et al 2007).

At a whole organism level the acute effects of cadmium exposure include increased mortality (Geffard *et al.* 2008). Following chronic cadmium exposure at sub-lethal concentrations, reduced feeding rate, reduction in phototactic behaviour and a decrease in neonate production has been reported (DeCoen and Janssen 1997; Michels *et al.* 2000; Geffard *et al.* 2008). The molecular effects of cadmium exposure have also been extensively investigated: acute sublethal exposure has been shown to have a significant effect on growth and energy metabolism within *Daphnia,* with reduced cellular energy allocation and a significant depletion of lipid reserves observed following cadmium exposure (Soetaert *et al.* 2007).

1.6 Strain variation within *Daphnia magna*

Toxicity testing in *Daphnia magna* is performed throughout the world in commercial and academic laboratories*. Daphnia* cultures are often established and then maintained in-house with little to no introduction of new animals, thus cultures are often reproductively isolated. Whilst there are established protocols pertaining to the rearing conditions i.e. media constitution, feed and supplements in addition to standardised testing procedures e.g. OECD 48hr acute immobilisation test, there is often little emphasis given to the strain of *Daphnia* used.

It is critical that the limitations of any given test are known in order for robust conclusions to be drawn from the data obtained (Oda et al. 2006). There are several bodies of work that suggest that the responses of different breeding cultures of *Daphnia magna* during toxicity testing may be significantly different and therefore have significant impact on the conclusions drawn from such studies.

In 1997 the OECD conducted a ring test into the reproducibility of their 21-day chronic toxicity testing procedure in *Daphnia magna* (OECD 1997). Amongst other objectives the ring test aimed to assess intra- and inter-laboratory variation with a total of 48 laboratories

across 17 countries participating. 7 distinct genotypes of *Daphnia* including “clone A” the most commonly used in Europe we exposed to three reference chemicals; cadmium chloride, 3,4-dichloroanaline (DCA) and phenol. Clone A appeared more sensitive to DCA. After further statistical analysis the authors concluded that for DCA, differences in EC_{50} values between laboratories could only be termed significant if they differed by greater than $20ugL^{-1}$ (OECD 1997).

In 2006 Oda *et al.* further investigated the differences in toxic response of 3 genetically distinct strains of *Daphnia magna* to several reference chemicals including heavy metals (Oda et al. 2006). Two strains were obtained from Japanese research institutions (NIES and Clone A) and the third from a commercial supplier, initial acute toxicity tests indicated a significant difference between EC_{50} amongst the three strains e.g. for copper sulphate: 13, 39 and 50 $\mu gCuL^{-1}$ (NIES, Commercial and Clone A, respectively). The authors concluded that the basis for the differences observed were predominantly genetic, though interestingly, Clone A that appeared to be most sensitive during the ring test (chronic) was the least sensitive to acute heavy metal toxicity. The authors further investigated these findings in a larger study involving seven genetically different strains of *Daphnia magna* from six laboratories across five countries, which were exposed to two juvenile hormone analogues; DCA and fenoxycarb (Oda et al. 2007). During initial acute toxicity tests the authors once again established that there were significant differences in sensitivity to the two compounds tested, with an EC_{50} ratio between the most and least sensitive strains of 2:1 for DCA and ~4.1 for fenoxycarb. Both compounds had been previously described to affect *Daphnia* reproduction, therefore the authors performed 21-day chronic reproductive testing (in line with OECD guidelines) and observed significant differences in sensitivity across the strains used (Oda *et al.* 2007).

Notable differences in the response of *Daphnia magna* to heavy metals has also been reported by a number of groups. In 2002, Barata *et al* investigated cadmium and zinc

toxicity to three distinct *Daphnia magna* genotypes obtained from different field populations within Spain and Germany (Barata *et al.* 2002). The authors performed feeding tests on 4-5 day old *Daphnia* in the presence of sub-lethal concentrations of cadmium (0.5-10 μgL^{-1}) and zinc (10 - 400 μgL^{-1}). They observed estimated EC_{50} (reduced feeding rate) of 2.2 and15.4 μgL^{-1} for cadmium, 399 and 521 μgL^{-1} for zinc for the most and least sensitive genotypes. Ward and Robinson (2005) observed differences in the acute toxicity of cadmium (48hr immobilisation test) in eight different cultures from academic institutions and commercial suppliers across the US. EC_{50} values ranged from 26μgL^{-1} to 120 μgL^{-1} (Ward and Robinson 2005). The inter- and intra-species differences in the acute toxicity of copper to different cladoceran species, including *Daphnia magna* has also been investigated using animals collected from several European sampling sites in addition to laboratory reared cultures (Bossuyt and Janssen 2005). The authors reported that values ranged from 5.3 to 70.6 μgL^{-1} across the species used, and more specifically within the *Daphnia magna* collected from different sites the EC_{50} varied between 26.8 and 53.2 μgL^{-1}.

Picardo *et al.* (2007) investigated the genetic variability among seven anonymous european laboratory cultures. The authors performed RAPD (random amplification of polymorphic DNA) fingerprinting and revealed that the strains clustered into two genetic groups (Picardo et al. 2007). The authors went on to perform acute immobilisation tests using two reference chemicals; potassium dichromate and zinc sulphate, in addition to two industrial effluent water samples. Results from these experiments demonstrated significant inter-lab differences in EC_{50}, with differences being most pronounced in response to effluent samples with a 10-fold difference between the most and least sensitive strains. This marked difference in response to a “real” situation where a complex mixture of contaminants exists lead to the same sample being classified as “toxic” using one culture

and “very toxic” in another, based on pre-defined thresholds (Picardo *et al.* 2007). This observation has broader implications within ecotoxicological testing as it is important to understand and predict how field populations may be affected by the presence of environmental contaminants.

An appropriate rationale for why there are differences between different culture sources for *Daphnia magna* was suggested by Haap and Köhler (2009). The authors suggest that selection pressures e.g. the presence of a given chemical within an environment, act on the population, giving rise to toxicant tolerant individuals or “locally adapted ecotypes” (Haap and Köhler 2009). Picardo *et al.* (2007) also postulate that genetic differences are the most probable explanation for the disparity in ecotoxicological responses observed amongst different cultures of both wild populations and laboratory cultures of *Daphnia magna* (Picardo *et al.* 2007).

The suggestion that *Daphnia magna* cultures originating from independent laboratories are genetically distinct and therefore as a consequence potentially exhibit different sensitivities to toxicants has significant implications. Cultures that are maintained in a laboratory would be expected to have descended from a small number of individuals and *as Daphnia* reproduce asexually, this genotype will in theory remain relatively unchanged. A laboratory population that is as homogenous as possible is desired to ensure reproducibility of experimental data, but the issue arises as to one, whether lab populations adequately represent the wild population of *Daphnia* and two, where the susceptibility of different strains varies greatly. These issues have grave potential to effect regulatory decisions. Whilst genetic differences have been proposed, it is important to state that the underlying biochemical and physiological functional differences between *Daphnia magna* cultures are not yet fully understood. This project aims to investigate this by examining the sensitivity of 10 strains of *Daphnia magna* obtained from different sources worldwide to heavy metal

exposure. Not only at the standard acute toxicity level but also at the biochemical level utilising a novel, non-targeted omics approach.

1.7 Metabolomics and ecotoxicology

The rapid development of new 'omics' technologies: genomics, transcriptomics, metabolomics and proteomics have revolutionised the manner in which biological questions are approached. Such techniques focus on a non-targeted, 'top down' approach, rather than focussing on a specific hypothesis and investigating a small number of specific reactions or variables (Garcia-Reyero and Perkins 2011). Metabolomics aims to characterise all of the endogenous, low molecular weight organic metabolites within a given biological sample e.g. tissue or organism (Bundy e*t al.* 2009; Han *et al.* 2008). The focus of metabolomics studies are often non-targeted, as such, novel relationships between metabolic changes and exposure may potentially be identified (Liu *et al.* 2006).This information can provide an insight into the metabolic and therefore physiological condition of the organism from which the sample is obtained at a particular moment in time (Liu *et al* 2006). The functional information that metabolomics provides can be important in understanding the biochemical processes involved in stress responses following toxicant exposure (Han *et al.* 2008).

Metabolomics is ideally suited to characterise the interaction of an organism with its environment as it offers relevant information about the biochemical state of the organism in question, which can in theory be linked back to phenotypic responses including sensitivity following acute exposure to toxicants (Bundy *et al.* 2009). Metabolomics offers an exciting avenue within ecotoxicology as it has the potential to characterise a 'metabolic fingerprint' indicative of toxicant induced stress responses from which information in terms of mode of action of the toxicant may be found. In addition, the relatively high-throughput manner in which experiments can be performed offers the potential to be applied as a screening tool

during the chemical toxicity testing that forms ecological risk assessment (Bundy *et al.* 2009).

1.8 Metabolomics platforms

The main aim of any metabolomics investigation is to gather comprehensive information about all of the low molecular weight metabolites within a given biological sample. In order to fulfil this aim the instrumentation used should be able to reliably detect, quantify and identify as many of these metabolites as possible. It is a formidable task given that there are many thousands of metabolites that are both structurally and physicochemically heterogeneous (Han *et al.* 2008). The ideal features of a metabolomics analysis platform include; high-throughput and unbiased, robust, reproducible and highly sensitive, in addition to having a wide dynamic range. Among metabolomics analysis platforms, the most widely used are Nuclear Magnetic Resonance (NMR) spectroscopy and Mass spectrometry (MS). NMR is robust and reproducible and with minimal additional sample preparation required, it is also a non-destructive technique (Lei *et al*. 2011). NMR is however, limited by its sensitivity and therefore unsuitable for use with *Daphnia magna* due to the small amount of biological material availiable (Taylor et al. 2009).

In contrast to NMR, MS is much more sensitive including the capacity to detect metabolites at fM concentrations. Front end separation techniques such as gas-chromatography (GC-MS) and liquid chromatography (LC-MS) can also be applied to reduce sample complexity and provide additional metabolite annotation in the form of retention times (Booth *et al.* 2011) However, these methods do have limitations; very few metabolites exist in the volatile, thermally stable states required for such techniques, thus additional sample preparation is often required which adds to analysis, preparation and costs, in addition to potentially introducing technical variation amongst the samples (Han et al. 2008; Lei et al. 2011). Direct Infusion Mass Spectrometry (DIMS), is a technique where

analysis is run directly on samples following limited preparation (Bergquist *et al.* 2002). It is limited to the analysis of compounds that are ionisable but can be conducted in both positive and negative ion modes in order to maximise the number of metabolites detected. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) is an ultra-high resolution DIMS technique (Barrow *et al.* 2005). It is suitable for use on large sample numbers due to its high-throughput nature. It is capable of high mass resolution (<1,000,000), therefore ions that differ in m/z ratio by only 5-6 decimal places may be resolved, leading to improved empirical formula calculations and metabolite identification (Barrow *et al.* 2005). FT-ICR MS is also capable of high mass accuracy <1ppm, but is limited by the finite dynamic range of the instrument in the detection of very high and low abundance metabolites within the same sample. SIM-stitch is an analysis method aimed at improving the dynamic range of the instrument (Southam *et al*. 2007). Briefly, data is collected in narrow overlapping spectra, each containing relatively few ions, and analysed by selected ion monitoring (SIM) system. These spectra are then “stitched” together to create a single continuous spectrum, with an approximately 22-fold greater dynamic range (southam *et al.* 2007). Based on the above, FT-ICR MS coupled with the SIM-stitching processing method has been selected as the platform for investigating the biochemical strain differences of *Daphnia magna* in this thesis, both at a basal level and following exposure to heavy metals.

1.9 MS data analysis

MS based metabolomics studies produce data that is multivariate in nature: where there are multiple simultaneous variables e.g. different metabolites within a number of different samples. This data requires analysis through multivariate statistical platforms, of which several are available, principal component analysis (PCA) was applied to the data within this thesis.

1.9.1 Multivariate statistical analysis – Principal Component Analysis (PCA)

Principal component analysis (PCA) is an unsupervised, projection technique that attempts to describe the overall variation in a sample data set, independently of knowledge about sample class e.g. treatment (Coen *et al.* 2008). It is a pattern recognition technique that allows for the visualisation of variation within a data set, based on the creation of PCA scores plots. The relative distances among individual samples in the scores plot represent the intrinsic similarities/differences between those samples i.e. samples that cluster together are similar in terms of the metabolites that are changing within those samples (Robertson 2005).

Sample data is broken down into “scores” and “loadings” in order to reduce the dimensionality of the dataset without a significant loss of information. Loadings summarise the variation between variables i.e. the intensity changes of individual m/z peaks within the data set. Peaks where intensity changes are large between samples are assigned high loadings values as these define the largest amount of variation within the data set. Scores summarise the variation between individual samples and are used to create PCA scores plots. PCA scores plots consist of two principal components (PCs) that describe the amount of variation between samples within the data set. The first principle component PC1 describes the largest amount of variation within the dataset. Different PCs may be used to create a plot depending on which ones best represent the variance within the data set. During interpretation of scores plots it is important to understand that they give no information with regards to mechanism or mode of action for the differences and similarities observed, in addition to having no statistically significant bearing (Coen *et al.* 2008).

1.9.2. Univariate analysis

Univariate statistical analysis may also be applied to metabolomics data, including standard *p*-tests (ANOVA and t-test) to identify metabolites that change significantly within the data set. However, for large data sets even a 0.05 significance value means that 5% of peaks could be assigned significance when they are not, leading to a large false discovery rate (FDR). The Benjamini and Hochberg (1995) method accounts for the FDR within large datasets and assigns an adjusted p-value (Benjamini and Hochberg 1995).

1.10 Research Aims

The aim of this thesis is to investigate strain to strain differences in *Daphnia magna* following exposure to the heavy metals cadmium and copper. With this larger goal in mind three separate aims were established:

1. To establish whether there are differences in the response of 10 different strains of *Daphnia magna,* following acute exposure of the heavy metals cadmium and copper as observed in other studies. This will be achieved through application of the OECD recommended acute immobilisation test across the 10 strains.

2. To investigate for the first time whether there are differences in the metabolism of these 10 different strains of *Daphnia magna* through the application of FT-ICR MS based metabolomics. In addition, to whether there are metabolic differences in the response of these strains following acute, sub-lethal exposure to cadmium and copper.

3. To develop targeted bioassays for use in *Daphnia magna* to investigate the biochemical and molecular mechanisms of cadmium and copper toxicity, with a view to applying these assays to assess a mechanistic basis for any differences in sensitivity observed.

These aims are individually addressed within Chapter 3, 4 and 5 of this thesis.

Chapter 2

Material and Methods

2.1 Culturing conditions for *Daphnia magna*

Daphnia magna where maintained in 2L beakers containing ca. 1200ml of OECD-recommended test media, modified by addition of 0.002 mgmL^{-1} sodium selenite (Table 2.1) (OECD 1998; OECD 2004). Animals were cultured in a semi-static system where media was renewed twice weekly, prior to use media was aerated for a minimum of 24 hrs. Cultures were initiated using neonates (<24 hrs old) of the third brood or later and maintained at a density of approximately 20 daphnia per beaker at a constant temperature of 18 ^{o}C with a light:dark photoperiod of 16:8hrs. Cultures were fed daily with a *Chlorella vulgaris* algal suspension (section 2.1.5) and a yeast supplement (section 2.1.3). The culture media was also supplemented with an organic seaweed extract (section 2.1.2).

Compound	Empirical formula	Concentration of stock solution	Volume of stock solution added to 4L of dH_2O
Calcium chloride	$CaCl_2 \cdot 2H_2O$	11.76 gL^{-1}	100ml
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	4.93 gL^{-1}	100ml
Sodium bicarbonate	$NaHCO_3$	2.59 gL^{-1}	100ml
Potassium chloride	KCl	0.23 gL-1	100ml
Sodium selenite	Na_2SeO_3	40 µgmL^{-1}	200µL

Table 2.1 Constituents of OECD modified culture media for *Daphnia magna*

2.1.1 Marinure nutritional supplement preparation

Marinure supplement, from a seaweed extract of *Ascophyllum nodosum* (Wilfrid-Smith Ltd, Oakley Hay, UK) was prepared by addition of ~10ml of extract to 1L of dH_2O. The optical density (OD) of a 1:10 dilution at 400nm within the range 0.76-0.84 is required. The prepared Marinure is then added to the culture medium at culture initiation and with every

subsequent media renewal; cultures less than 7 days old received 3ml and those greater than 7 days, 4ml. The Marinure stock should be replaced at least every 6 months.

2.1.2 Preparation of *Saccharomyces cerevisiae* (Yeast) Solution

Daphnia were fed daily with 0.5ml of *Saccharomyces cerevisiae* (yeast) supplement solution per culture beaker. A 100mgL^{-1} stock was prepared by addition of 10mg of dried yeast (Sigma-Aldrich, UK) to 100ml of dH_2O. The stock was stored at 4 ^oC and renewed fortnightly.

2.1.3 Culturing conditions for *Chlorella vulgaris*

The unicellular green alga *C.vulgaris* was cultured for the use of Daphnia feed. Six cultures were maintained in a sterile system in Bold's basal medium (BBM) (Table 2.2). The system was exposed to a constant source of photosynthetic light and aeration. BBM was prepared in 5L batches and autoclaved before use.

Number	Compound	Empirical formula	Concentration of stock solution	Volume of stock prepared	Volume of stock added to prepare 5L of BBM
1	di-Potassium hydrogen orthophosphate	K_2HPO_4	7.5 gL^{-1}	1L	50ml
2	Potassium di-hydrogen orthophosphate	KH_2PO_4	4.93 gL^{-1}	1L	50ml
3	Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	7.5 gL^{-1}	1L	50ml
4	Sodium nitrate	$NaNO_3$	25 gL^{-1}	1L	50ml
5	Calcium chloride	$CaCl_2 \cdot 2H_2O$	2.5 gL^{-1}	1L	50ml
6	Sodium chloride	NaCl	2.5 gL^{-1}	1L	50ml
7	EDTA tetrasodium salt Potassium hydroxide	EDTA - Na_4 KOH	50 gL^{-1} 31 gL^{-1}	100ml	5ml
8	Ferrous Sulphate conc. Sulphuric acid	$FeSO_4 \cdot 7H_2O$ H_2SO_4	4.98 gL^{-1} 10mlL^{-1}	100ml	5ml
9	Boric acid	H_3BO_3	11.42 gL^{-1}	100ml	5ml
10	Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	14.12 gL^{-1}	25ml	500µl
11	Manganese chloride	$MnCl_2 \cdot 4H_2O$	2.32 gL^{-1}	25ml	500µl
12	Copper sulphate	$CuSO_4 \cdot 5H_2O$	2.52 gL^{-1}	25ml	500µl
13	Cobaltous nitrate	$(Co(NO_3)_2 \cdot 6H_2O$	0.8 gL^{-1}	25ml	500µl
14	Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	1.92 gL^{-1}	25ml	500µl

Table 2.2. Constituents of Bold's Basal Medium (BBM) for the culture of *C.vulgaris*

2.1.4 Preparation of algal feed

Algal feed was prepared from the cultured *C.vulgaris* through removal of a known volume of algal suspension from the culture. The OD of a 1:10 dilution was obtained, of which an OD of 0.8 at 440nm is desired. To achieve the correct density, the suspension was concentrated through centrifugation at 3000 rpm for 30 min and resuspended using dH_2O. Following preparation the feed is stored at 4 ^oC and renewed fortnightly. *Daphnia* were fed varying amounts in an age proportionate manner; those <2 days old received 1ml, 3-7 days old 1.5ml and cultures 8+ days old were given 2ml.

2.2 *Daphnia magna* strain information

Daphnia magna cultures were kindly donated by a variety of UK and international research institutions, an industrial company[1], one commercial supplier, in addition to two cultures from our own lab that were separated approximately 4 years ago and were designated B1 and B2 (Table 2.3). Herein the different cultures will be referred to as separate 'strains'. Immediately upon arrival new *Daphnia* strains were transferred to OECD media and the culture conditions described previously in section 2.1. A period of acclimatisation of at least four generations was observed prior to any testing being carried out.

Institution	Country	Strain name
Industrial	UK	IS
Blades Biological - Commercial supplier	Kent, UK	BD
Okazaki Institute for Integrative Science, National Institute of Natural Sciences	Okazaki, Japan	NS
Universitá del Piemonte Orientale	Alessandria, Italy	UPO
University of Antwerp	Antwerp, Belgium	AW
University of Birmingham	Birmingham, UK	B1
University of Birmingham	Birmingham, UK	B2
University of California, Berkeley	California USA	BK
University of Reading	Reading, UK	RD
US Environmental Protection Agency (EPA)	Minnesota, USA	EPA

Table 2.3. Location and institution from which *Daphnia magna* strains originated

[1] For confidentiality reasons the source of this strain cannot be revealed

2.3 Acute toxicity exposures

2.3.1 EC_{50} - acute immobilisation test

The acute toxicity of cadmium and copper was determined using a standard 48hr immobilisation test in order to establish an EC_{50}. Each test was performed in accordance with OECD guidelines, as detailed in the OECD guidelines for testing of chemicals No. 202 (OECD 2004) briefly:

Replicates of 10 neonates (<24hrs old) were exposed to a range of concentrations in 200 ml of culture media (section 2.1) in the absence of food or additional supplements. Metals were administered as their respective salts; $CdCl_2 \cdot H_2O$ and $CuSO_4 \cdot 5H_2O$ (Sigma-Aldrich) , doses were based on ionic metal concentrations and ranged between 2.5 and 70 μgL^{-1} Cu^{2+} and 125 and 3000 μgL^{-1} Cd^{2+}. $1gL^{-1}$ stocks were made up for each metal with the copper stock being further diluted to give 100 and 10 mgL^{-1} working stocks. Each dose was performed in quadruplicate in addition to a control, untreated group also in quadruplicate.

Exposure beakers were maintained under the same environmental conditions as detailed in section 2.1, following 48 hr the media in each beaker was gently agitated, animals that did not resume swimming after ~15 seconds were considered to be immobilised, as detailed in OECD test guidelines (OECD 2004).

2.3.2 Sub-lethal exposure for metabolomics analysis

The acute 48 hr EC_{50} was used to inform on a single, sub-lethal dose for each metal: 100 μgL^{-1} Cd^{2+} and 500 ngL^{-1} Cu^{2+}. In the interests of reducing technical variation a single stock of each metal was used for all the exposures.

Animals for use in metabolomics experiments were raised under the same conditions as described in section 2.1. 14 day old adults were then transferred to exposure beakers and assigned at random to 3 treatment groups (control, copper and cadmium treated), n = 10.

Exposures were performed in 200 ml of media (section 2.1) in the absence of food or additional supplements and maintained under the same environmental conditions as described in section 2.1. Following 48 hr, adults were captured individually via filtration through a fine mesh and using a fine paint brush, were immediately transferred to a Precellys™ homogenisation tube (Stretton Scientific Ltd, UK) and flash frozen in liquid nitrogen. Samples were then stored at -80 °C for a period of no more than a week before metabolites were extracted as detailed in section 2.4.

2.4 Metabolite extraction

Following exposure (section 2.3.2) each single whole adult sample was subjected to a biphasic extraction process in order to obtain samples for MS analysis, using a 2:2:1.8, methanol:chloroform:water solvent ratio as described previously (Taylor et al. 2009). 320 µL of ice cold methanol (HPLC-grade, Fisher Scientific, UK) and 128 µL of ice cold H_2O (HPLC-grade, J.T. Baker) were added to each Precellys™ tube containing the whole adult sample. Samples were then homogenised for 2x 10 second bursts at 6400 rpm using a Precellys™ 24 homogeniser (Stretton Scientific Ltd, UK). The homogenate was transferred to a pre-cooled, 1.8 ml glass vial on ice and a further 160 µL of HPLC-grade H_2O and 320 µL of chloroform (HPLC-grade Fisher Scientific, UK) (both ice-cold) was added, the later using a glass Hamilton syringe (Hamilton Bonaduz AG, Switzerland). After vortexing, each vial was left on ice for 10 minutes and then centrifuged at 4000 rpm for 10 minutes at 4 °C. By this process the sample will separate into two layers, an aqueous, polar and organic, non-polar fraction. 300 µL of the polar fraction was removed with the use of a Hamilton syringe, split into two aliquots and placed in 1.5ml eppendorf tubes. 200 µL of the non-polar fraction was removed and placed in a 1.8 ml glass vial. Care was taken to avoid disturbing the interface region between the two layers which contains protein debris and syringes were washed in HPLC grade methanol or chloroform between each extraction to

avoid cross contamination of samples. Each 150 µL of the polar aliquot was dried with the use of a SPDIIV SpeedVac concentrator (Thermo Scientific). Non-polar samples were dried with the use of a nitrogen sample concentrator (Techne). Following extraction, all fractions were stored at -80 ^{o}C.

Extract blanks containing no biological material and quality control (QC) samples from approximately 30, 14 day old adults, were extracted in the same manner. These samples are required for downstream data processing of the spectral data (section 2.6).

2.5 FT-ICR Mass spectrometry

One of the dried polar aliquots of each sample was resuspended for analysis in negative ion mode; the second aliquot was retained for analysis in positive ion mode but was not performed in this thesis. A resuspension solvent consisting of 80:20 methanol:water (both HPLC grade) with 20mM ammonium acetate was prepared and 30 µL was added to each sample. After thorough vortexing the samples were centrifuged at 15,000 rpm at 4 ^{o}C for 15 minutes in order to pellet out residual protein material from the extraction process. Taking care not to disturb the pellet, 5 µL aliquots were loaded into a 384-well plate in quadruplicate using an automated multi-dispensing pipette. QC samples were resuspended in the same manner, pooled and aliquoted out to be run across the final MS plates dispersed between samples along with extract blanks. All plates were pre-cooled, loaded on ice and immediately covered in 20 µm heat-sealing foil and sealed using a Thermo plate sealer (Agbene, UK) to minimise sample evaporation.

Samples were analysed using a 7-T FTICR Mass spectrometer (Thermo Scientific, Bremen, Germany) using a direct infusion nanoelectrospray ionisation source (Triversa, Advion Biosciences NY) under conditions described previously (Taylor et al. 2009).

2.6 MS data processing

Data was acquired as transients and processed using a custom written SIM-stitching method (Southam *et al.* 2007). Briefly; data in the 70-590 m/z window collect as 7 separate 100 m/z windows with a 30 m/z overlap. At this stage one replicate from each sample was discarded from further analysis, unless there was sufficient reason to discard another e.g. poor spray stability, the first replicate from each sample was discarded. The SIM-stitch algorithm was applied with the following parameters: peaks with signal-to-noise (SNR) ratio of < 3.5 were excluded from further analysis as noise. Remaining peaks were calibrated based on an external calibrant list (Appendix A.). SIM-windows were then stitched together to create a single continuous spectrum for each of the three replicates for each sample, generating an individual peak list consisting of m/z and intensity values.

A two-stage filtering process was then employed to remove highly variable peaks (Payne *et al.* 2009). Within replicate filtering (of each individual sample); peaks that do not appear in at least 2 out of the 3 sample replicates were removed, a cut off of 1.5 ppm was chosen for the maximum distance between peaks along the m/z axis in different replicates that were considered to arise from the same metabolite. This filtering stage generates a single list of peaks for each biological sample.

A second sample filtering stage is then applied to these biological peak lists. By this process peaks that do not occur within a certain percentage of samples within the whole dataset are discarded from any further analysis, peaks within different samples that were less than 2 ppm apart along the m/z axis were considered to arise from the same metabolite. Also all peaks that occurred in the extraction blank samples were removed from the peak list to ensure that only true biological peaks are retained.

The above filtering process generates a final peak or ‘x’ matrix of intensity values, with each row corresponding to individual samples and each column representing individual

m/z values (peaks). Due to the nature of this sample filter the x matrix will have missing values where peaks did not occur within a subset of samples, samples with greater than 50% missing values were then removed to prevent the influence of these samples on further analysis.

In order to minimise the effects of highly variable peaks the entire data set is then normalised using probabilistic quotient normalisation (PQN), which individually assesses all peaks within the dataset that do not have missing values and assigns a normalisation scaling factor, or median scaling factor which is then applied across the whole data set including those with missing values (Dieterle *et al.* 2006). Further variation was removed based on individual peak relative standard deviation (RSD), peaks with an RSD of greater that 30% were also removed from further analysis.

In order for further (multivariate) analysis missing values are then assigned an estimated intensity based on k-nearest neighbour imputation (KNN) as described previously; where K = 5 (Hrydziuszko and Viant 2009). Giving rise to a normalised complete peak matrix with corresponding peak intensities for every sample within the data set.

A generalised-log (g-log) transformation was then applied in order to reduce the effect of highly abundant peaks where technical variation is more pronounced, and may impact on downstream multivariate analysis (Parsons *et al.* 2007). This transformation normalises the technical variance within these peaks by reducing their intensity and concomitantly increasing the intensity of low abundance peaks (Dettmer *et al.*2007). A g-log transformation parameter or λ is optimised using the QC samples i.e. the same biological sample and then applied across the entire data set.

2.7 Statistical analysis

2.7.1 Multivariate statistics

Multivariate statistics was applied to the final g-logged MS data set (section 2.6). PCA was employed within this thesis using MATLAB® software (MATLAB® v 7.14; Eigenvector Research, USA). Within each model the number of PCs accounted for a minimum of 60% of the total variance within the data set but varied depending on the model applied. Scores plots of the lowest number PCs that accounted for the greatest amount of variance within the samples were generated using the same software.

2.7.2 Univariate statistics

All data was tested for normal distribution based on Anderson-Darling test for normality using Minitab® Statistical Software v.15 (Minitab Inc.) (Anderson and Darling 1952). The appropriate parametric or non-parametric statistical analysis was then applied. The degree of association between two sets of normally distributed data was assessed through calculation of Pearson's correlation coefficient 'r' using Minitab®. Differences between the means of two groups of normally distributed data were statistically analysed using student's t-test Minitab®. Student's t-test and ANalysis Of Variance (ANOVA) were also applied to the mean PC scores data (section 2.7.2.) depending on the number of sample classes defined, using MATLAB®. Grubb's test for outliers within data was performed using GraphPad Grubb's outlier calculator (graphpad.com Software, Inc.).

Chapter 3

Investigating the response of 10 strains of *Daphnia magna* to acute cadmium and copper toxicity using the OECD acute immobilisation test

3.1 Introduction

The acute immobilisation test and the generation of an EC_{50} is a standard endpoint during toxicity testing, required near ubiquitously by regulatory bodies including REACH (Foth and Hayes 2008). Standard protocols are available and adopted to ensure that experiments performed throughout different laboratories are as comparable as possible (OECD 2004). As previously discussed in section 1.6 there have been numerous studies reporting disparities in the response of different strains of *Daphnia magna* to the acute immobilisation test; both between strains originating from wild populations but most notably variation in the response of cultures from different laboratories (Oda *et al.* 2006; Oda *et al.* 2007; Ward and Robinson 2005; Barata *et al.* 2002). In this chapter the response of 10 strains of *Daphnia magna* during acute exposure to the heavy metals cadmium and copper was investigated through the application of the OECD acute immobilisation test (OECD 2004).

3.2 Materials and methods

Immobilisation experiments for both metals were conducted as described in section 2.3.1; every care was taken to ensure that the test was performed in the same manner across all of the strains used.

Initial exposures were performed to determine a suitable range of doses for full EC_{50} experiments. These experiments were performed using B1 and B2 neonates early in the course of this project as these strains required no acclimatisation to culture conditions. Based on these initial investigations a “standard” range of doses was chosen: 750 - 2250 μgL^{-1} for cadmium and 20 - 50 μgL^{-1} for copper. A critical dose study (top and bottom doses of this range) within each strain was performed and the dose range for the full EC_{50}

exposure was then adjusted accordingly (Tables 3.1 and 3.2). Final dose ranges were between 100 and 3000 μgL^{-1} for cadmium and 2.5 and 70 μgL^{-1} for copper, dependent on strain sensitivity.

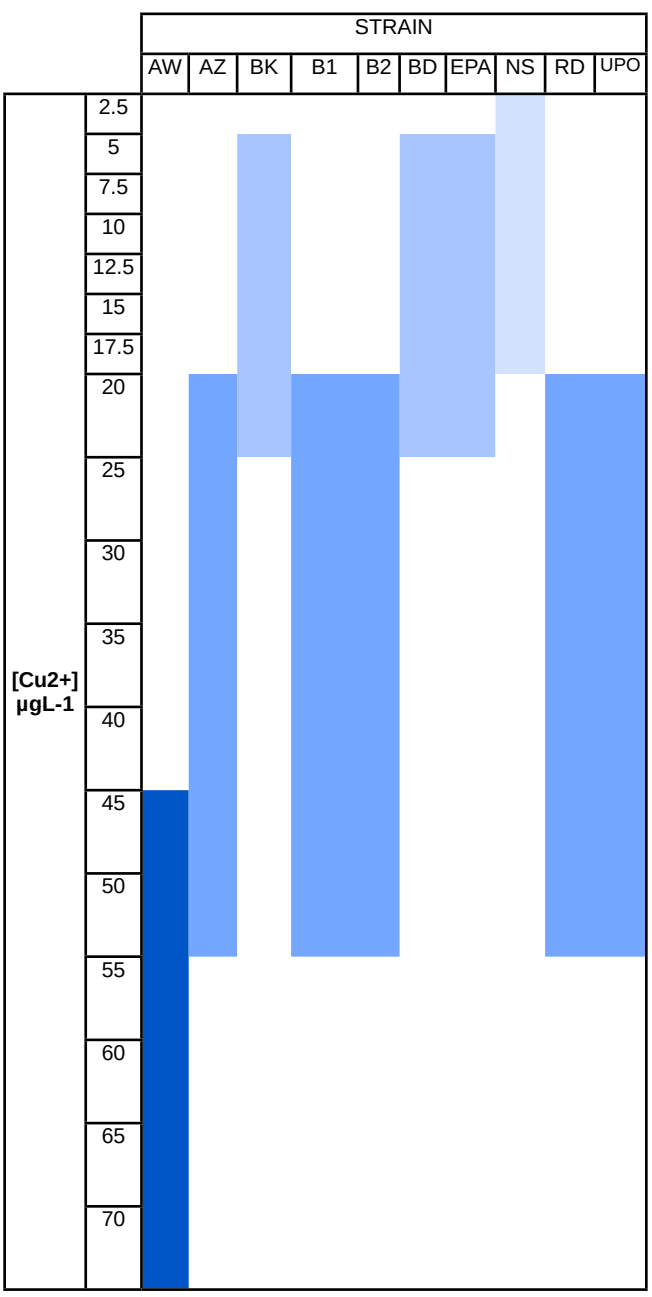


Table 3.1. Dose ranges used for copper acute immobilisation test across 10 strains of *Daphnia* magna in order to generate an EC_{50}. Doses ranged from 2.5 to 70 μgL^{-1} and are indicated by shaded blue boxes for each strain.

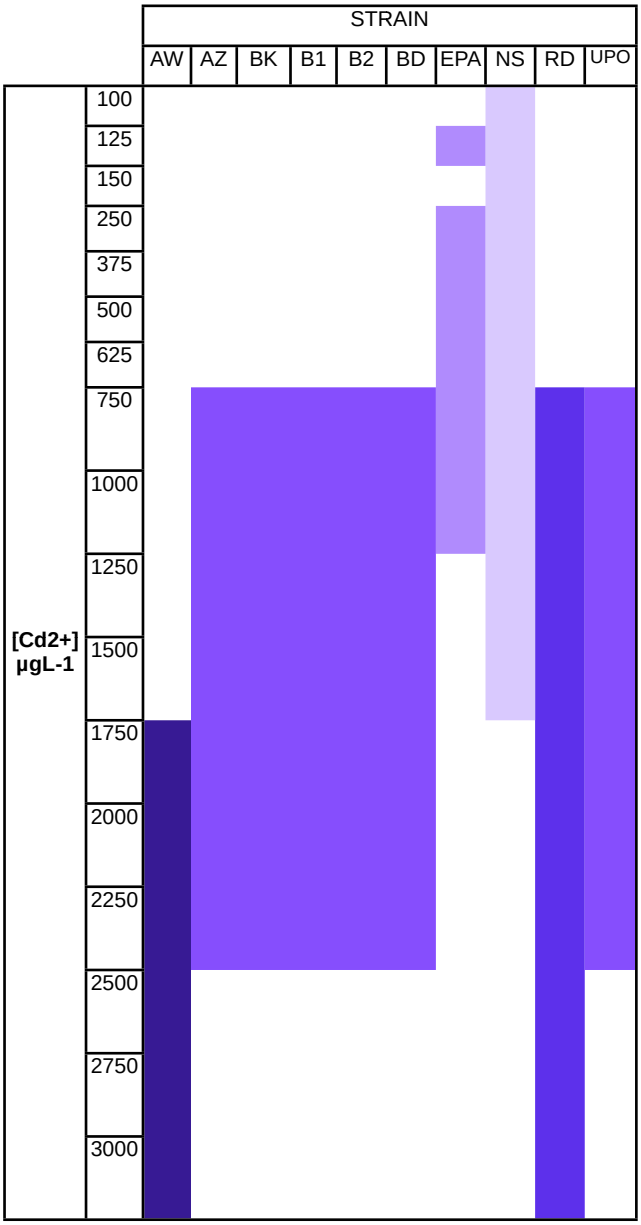


Table 3.2. Dose ranges used for cadmium acute immobilisation test across 10 strains of *Daphnia* magna in order to generate an EC_{50}. Doses ranged from 100 to 3000 μgL^{-1} and are indicated by shaded purple boxes for each strain.

Raw immobilisation data was analysed using the Trimmed Spearman-Karber (TSK) Method (TSK v1.5, U.S. EPA) to generate an EC_{50} for each strain and each toxicant. Probit analysis (RASW Statistics 18, SPSS Inc., IBM) was also applied to the same data. Paired t-testing revealed that the EC_{50} values obtained via the two separate methods were not statistically different: for clarity only the TSK EC_{50} values are reported and discussed herein.

3.3 Results

From the data reported in figure 3.1 the strain that appears to be the least sensitive to copper exposure is AW with the highest EC_{50} = 59.200 μgL^{-1} (95% conf. = 56.750 to 61.250), while the strain that displayed the most sensitivity to treatment was NS with the lowest EC_{50} = 4.770 $\mu gL^{-1,}$ (95% conf. = 4.170 to 5.450). The response of the B1 and B2 strains appear to be similar with EC_{50} values being relatively close together at 41.890 μgL^{-1} and 41.190 μgL^{-1} respectively, in addition to a considerable overlap in the 95% confidence intervals. There appears to be a clear separation amongst the strains in terms of response, with a few relatively sensitive strains i.e. NS and EPA, BK. The majority of the remaining strains e.g. B1 and B2, IS, RD, UPO, BD have similar EC_{50} values and appear to form a subset of strains with a similar sensitivity. The AW strain appears cosiderably more tolerant to acute copper exposure than the other 9 strains with an EC_{50} considerably larger than the rest (59.200 μgL^{-1}). Moreover there was a 12-fold difference between the EC_{50} of NS the most sensitive and AW the least sensitive strain.

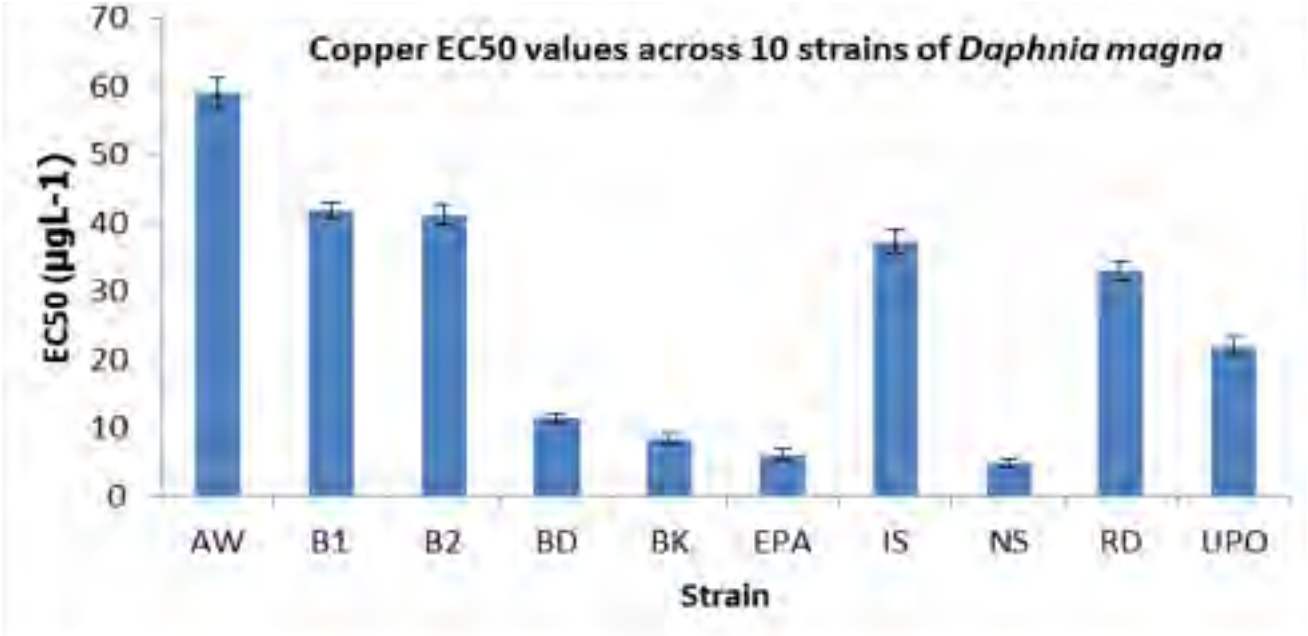


Figure 3.1. Histogram showing $EC_{50}(\mu gL^{-1})$ across 10 different strains of *Daphnia magna* following treatment with copper. EC_{50} calculated using TSK method, error bars denote 95% confidence intervals.

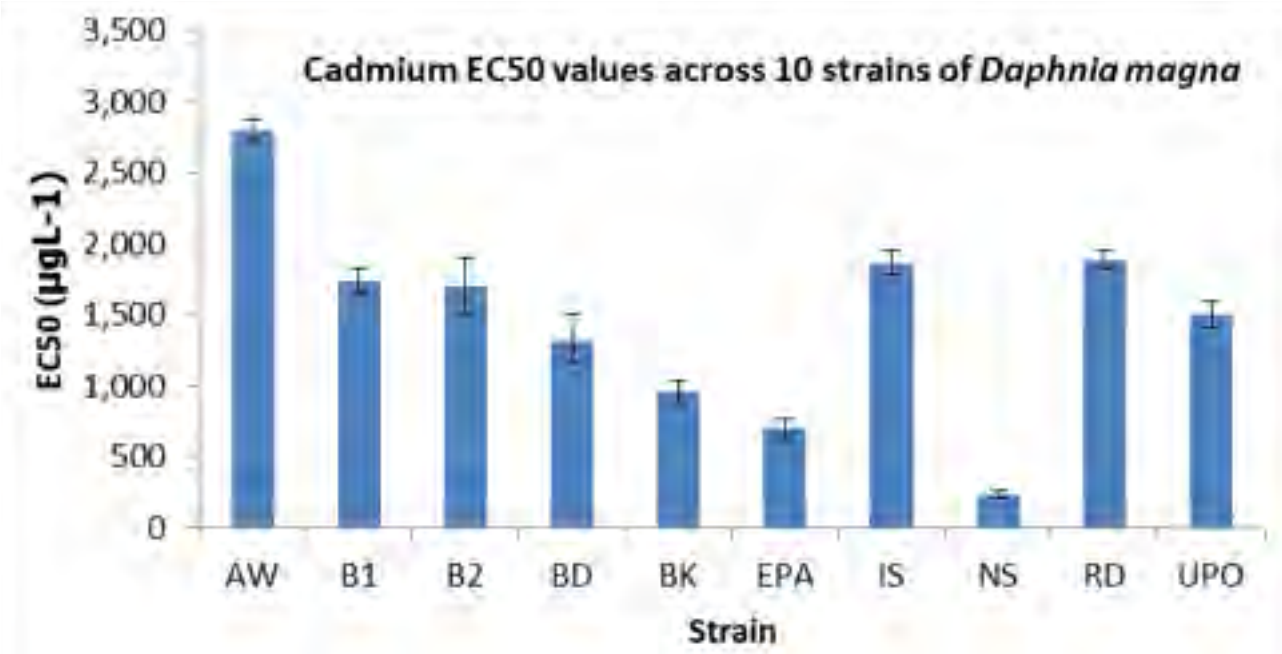


Figure 3.2. Histogram showing $EC_{50}(\mu gL^{-1})$ across 10 different strains of *Daphnia magna* following treatment with cadmium. EC_{50} calculated using TSK method, error bars denote 95% confidence intervals.

Based on the EC_{50} calculated (figure 3.2), the least sensitive strain to cadmium treatment was again AW, EC_{50} = 2800.560 μgL^{-1} (95% conf. = 2724.86 to 2878.320), similarly, the most sensitive strain was again NS with the lowest EC_{50} = 234.380 μgL^{-1} (95% conf. = 208.810 to 263.000). As with copper exposures there was again, almost a 12 fold difference between the highest and lowest EC_{50} values observed. Similar to the effects observed following copper treatment, the 10 strains used appear to display very different responses to acute cadmium stress. Once again there is a clear grouping of more sensitive strains with lower calculated EC_{50} values around or below 10 μgL^{-1}, including BK, EPA, NS and BD. Once again, the Birmingham strains (B1 and B2) (EC_{50} =1742.510 μgL^{-1} and 1695.650 μgL^{-1}) appear to display comparatively little difference in sensitivity to cadmium and form a core of five strains with IS, RD and UPO, which appear to be much more resilient. AW once again stands out as a particularly in-sensitive in comparison with the other 9 strains.

Following the observation that most and least sensitive strains to acute cadmium and copper exposure were the same between the two treatments, the EC_{50} values for each strain across the two toxicants were plotted in figure 3.3.

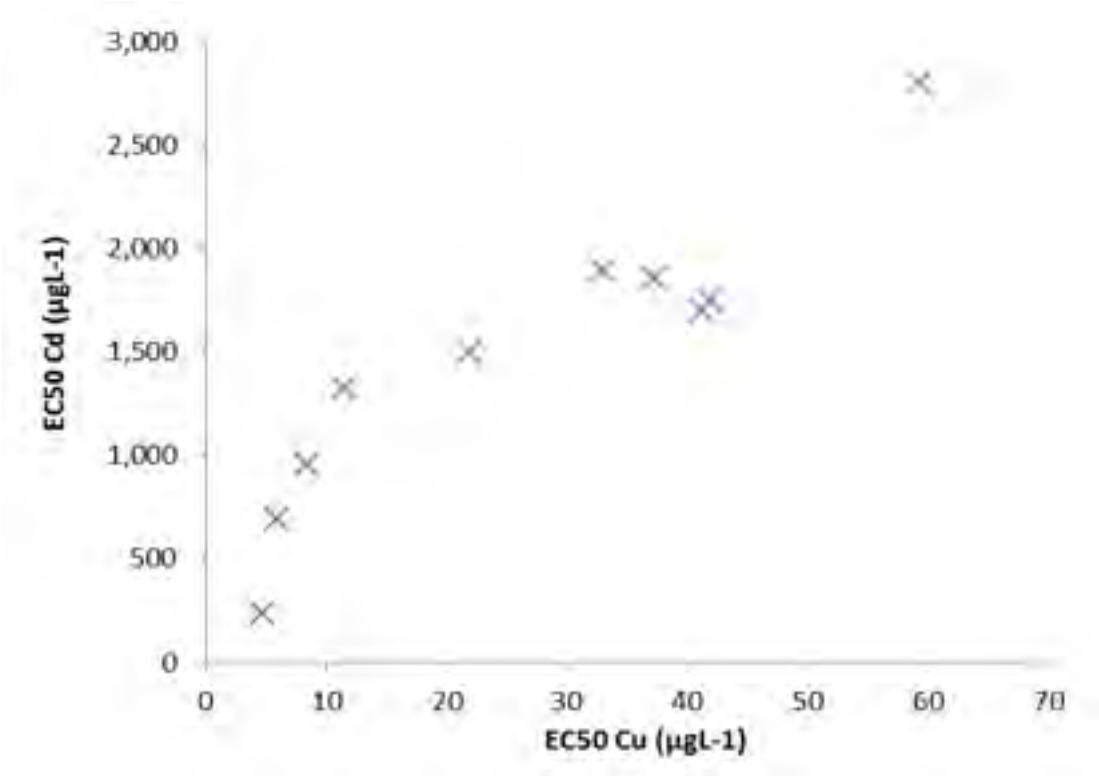


Figure 3.3. EC_{50}(µgL^{-1})Cd versus EC_{50}(µgL^{-1})Cu across 10 different strains of *Daphnia magna*. EC_{50} calculated using TSK method as before. r = 0.928 (p = <0.05).

Data was first tested for normality (section 2.7.1) and the correlation between the two EC_{50} values was then assessed through the calculation of a Pearson's correlation coefficient (r). In this instance r = 0.928 (p = <0.05), indicating a strong linear dependence between the EC_{50}, and therefore sensitivity to cadmium and copper observed across all the strains.

3.4 Discussion

As reported in section 3.3 there were marked differences observed between the EC_{50} values obtained across the 10 strains used following separate exposures to the heavy metals cadmium and copper. A total 12-fold difference was observed between the most and least sensitive strains (NS and AW respectively). Furthermore a clear separation between strains may be observed in terms of a subset that appear particularly sensitive and AW that appears relatively insensitive.

Moreover, there was a clear correlation between the sensitivity to both toxicants, based on current literature the heavy metals cadmium and copper are thought to share similar mechanisms of toxicity, including an induction of oxidative stress. This common mechanism of toxicity may account for the correlation in sensitivity between the treatments observed in figure 3.3. These results confirm observations previously made within scientific literature that reported significant differences in the response of different strains of *Daphnia magna* to toxicant stress.

There are several possible reasons as to why such different acute sensitivities were observed between these two strains. The differences in EC_{50} values reported in the scientific literature, for experiments conducted in different laboratories using *Daphnia magna* exposed to the same toxicant, are likely to be due to several factors including the culture conditions. Most notably, the culture media used is likely to have a significant effect on metal speciation and thus the bioavailability of free metals ions, in addition to differences in the protocols used and individuals performing the experiments (Christiansen *et al.* 2011). During the course of this project all possible measures were undertaken to ensure that experiments, although conducted on separate days were as comparable as possible, eliminating variables such as different culture conditions and the use of the same metal salt stock throughout the experiments. It is therefore unlikely that the physical manner in which these experiments were performed have given rise to such differences in sensitivity observed here.

It is possible that the differences in response are as a result of the change in culture conditions following the transfer of these organisms from their original institution to our own. Little is known about the time it takes for *Daphnia* to adapt to new conditions. It is therefore possible that strains that came from institutions using culture media and conditions very different from our own were at a disadvantage. At the time of exposure these animals may have been weakened by the environmental change and therefore,

showed a greater sensitivity to cadmium and copper as occurred with the NS, EPA and BK strains. Strains were however, acclimatised for a minimum of four generations before EC_{50} experiments were performed in an attempt to minimise such effects. In order to address this possibility, it would be prudent to fully repeat the exposures in exactly the same manner following a longer time period e.g. 3 or 6 months. Such a study however, was beyond the time limits of this project and therefore cannot currently be addressed.

A third option remains that may account for the differences in sensitivity observed between the two strains. It is likely that the differences are not, in fact, artefacts of poorly acclimatised strains or the manner in which they were performed, but is due to the intrinsic differences between the strains themselves. It has already been established that there are genetic differences both within laboratory and wild populations of *Daphnia magna,* and that these genetically different strains manifest with different acute toxicity responses to toxicants (Bossuyt and Janssen 2005; Oda *et al.* 2007). It is therefore not unreasonable to suggest that the strains used within this project may be genotypically different from each other and perhaps consequently differ in their response following heavy metal exposure. Due to the limitations of the acute immobilisation test, little can be established in terms of a mechanistic basis for the toxicity observed. Therefore, it is beyond the limits of this experiment to assign any putative reasons for the differences in sensitivity to cadmium and copper that were observed. Nevertheless it is a major concern that effectively the same exposure performed in different labs e.g. using different strains (irrespective of culture conditions etc.) may give different results for this standard test. If true, the implications for environmental risk assessment and legislation are profound. Testing carried out in one lab, using a particular strain of *Daphnia* may report an EC_{50} following cadmium or copper exposure below a pre-defined toxicity threshold, whilst a different laboratory may report an EC_{50} above the same threshold following exposure to the same chemical. In order for risk

assessment and consequent legislation to be robust the results from independent laboratories must agree.

Chapter 4

Application of FT-ICR MS to investigate the metabolic differences between 10 strains of *Daphnia magna* and in their response to sub-lethal cadmium and copper exposure

4.1 Introduction

Marked differences in the sensitivity of 10 strains of *Daphnia magna* were observed during the acute immobilisation tests performed in Chapter 3, confirming the findings reported in other studies within the scientific literature (section 1.6). Due to the nature of this test no additional information including a mechanistic basis for the toxicity observed can be elucidated beyond the generation of an EC_{50}. However, in order for robust conclusions to be drawn, including regulatory decision making it is important to know not just if these differences occur but also why. Whilst genetic differences have been postulated as giving rise to the differences in sensitivity observed, little in terms of a mechanistic basis has been elucidated. In this chapter a non-targeted MS-based metabolomics study using 10 strains of *Daphnia magna* following exposure to a single, sub-lethal dose of the heavy metals cadmium or copper was performed. With the aim to investigate whether a metabolic difference may account for the acute toxicity responses observed.

4.2 Materials and methods

Animal exposures were performed as described in section 2.3.2, due to the capacity limitations of our current *Daphnia* facility exposures were conducted in batches (Table 4.1.) across a 4 week period. All exposures were initiated at the same time of day for each batch.

Strain	Batch	Exposure week	Extraction week
UPO	A	11 Jun 2012	18 Jun 2012
EPA			
B2			
BD	B	18 Jun 2012	25 Jun 2012
RD			
BK			
IS	C	25 Jun 2012	2 Jul 2012
AW			
B1	D	2 Jul 2012	2 Jul 2012
NS			

Table 4.1. Batch identification of metabolomics exposures and extractions for 10

Following exposure, samples were stored at -80 ^{0}C until metabolite extraction (section 2.4.). To improve the manageability of this study extractions were also performed in batches over several days (Table 4.1), samples were randomised throughout the extraction process (e.g. between treatments and strains within each batch) to minimise the effects of technical variation. To account for sporadic death during exposures and errors during the extraction process final MS analysis was performed with an n = 8. All samples including blanks and QCs were run in quadruplicate as described in section 2.5. Due to malfunctions in the ion source equipment (Triversa) and FT-ICR nitrogen source, the total 281 samples (240 biological samples, 35 QCs and 6 blanks) were ran across 6 partially-full 384-well plates (Appendix B.).

Data was acquired via the use of the SIM-stitch method and processed as described in section 2.6. A 50% sample filter was applied resulting in an ‘x’ matrix of 1,870 peaks. RSD filtering lead to the removal of 353 peaks, resulting in a final data set of 1517 peaks with a median RSD of 12.75%. The g-log transformation parameter (λ), optimised using QC

samples was defined as $\lambda = 1.5428 \times 10^8$. Based on peak m/z value, possible empirical formula and putative identity of corresponding metabolites was determined using custom written MI-PACK algorithm (Weber and Viant 2010).

4.3 Results

Following data processing as described in section 2.6, multivariate data was analysed through the calculation of PCA models (section 2.6). The technical variation across all samples was first assessed. Figure 4.1(A) shows that technical replicates of the pooled QC sample cluster tightly together across PC1 and PC2 with no drift during the course of the experimental run. This indicates that technical reproducibility of samples within the instrument during this experiment was very high. Results shown in figure 4.1(B) indicate there is no clustering between samples ran on the same plate, with samples located sporadically across PC1 and PC2. These results suggest that technical variation between different plates does not underlie variation within the data set. The variation between extraction and exposure batch was also assessed and no significant clustering of samples within the same batch was observed (data not shown). The results shown in figure 4.1 show that the QC samples were technically reproducible, and were thus removed from further analysis.

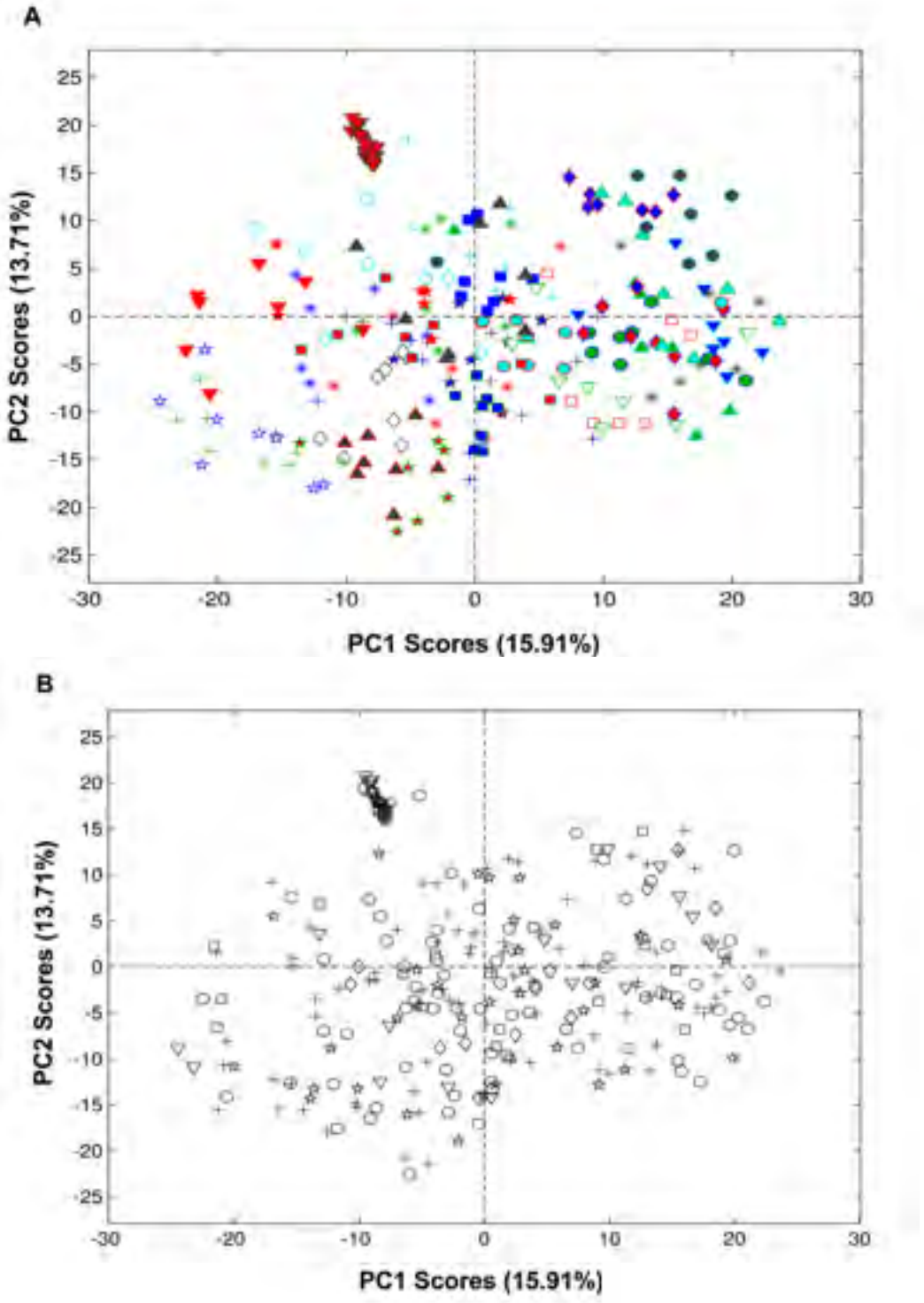


Figure 4.1 PCA scores plot from analysis of FT-ICR MS data of samples of whole adult *Daphnia magna*. (A) Biological samples grouped individually by treatment, within each strain (31 groups), QCs are shown as red triangles with black boarder. (B) Biological samples grouped by order of MS Plate; triangle = 1, star = 3, cross = 4, square = 5, circle = 6. Both plots show PC1 against PC2

Figure 4.2(A) shows no clear clustering or separation of any groups i.e. treatments, the 30 separate groupings of samples within this model are too numerous to make any sensible interpretations. Based on this observation, a PCA scores plot of samples grouped according to the strain of *Daphnia magna* they originated from were produced (figure 4.2(B)).There is clear clustering of samples originating from the same strain, in addition to separation between particular strains, the greatest separation was along PC1 that accounted for 17.28% of total variance. Some separation along PC2 (8.85% of total variance), between B1, B2 and RD samples was also observed. Following grouping of samples according to treatment (figure 4.2 (C)), there was no clear clustering of samples exposed to individual treatments.

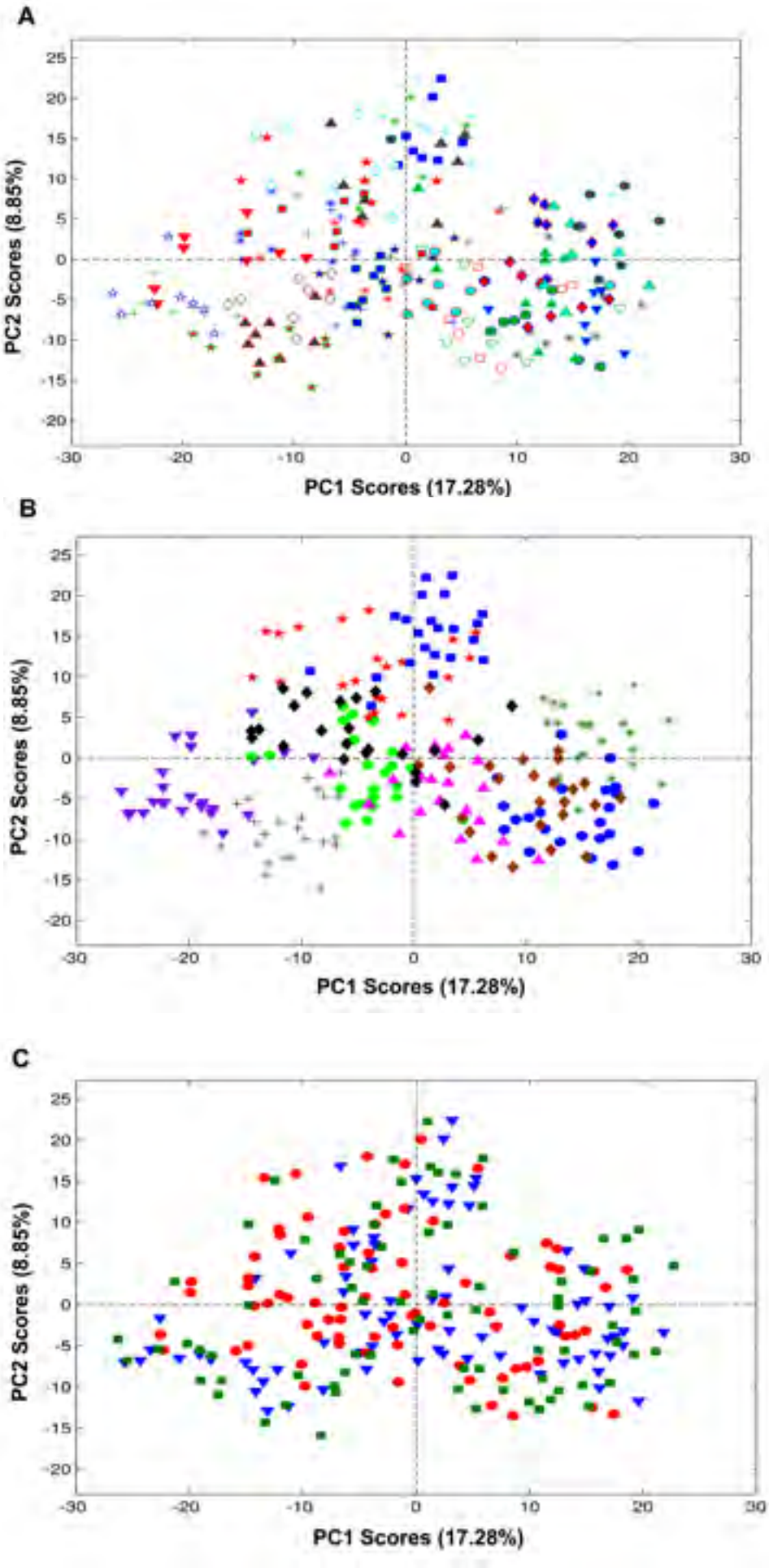


Figure 4.2 PCA analysis of FT-ICR MS data of samples of whole adult *Daphnia magna.* (A) Biological samples grouped individually by treatment within each strain (30 groups). (B) Biological samples grouped based on *Daphnia magna* strain, irrespective of treatment; purple triangle = AW, red star = B1, blue square = B2, black cross = BD, blue circle = BK, brown diamond = EPA, green circle = IS, pink triangle = NS, black diamond = RD, green star = UPO. (C) Biological samples grouped according to treatment; red circle = cadmium, blue triangle = control, green square = copper.

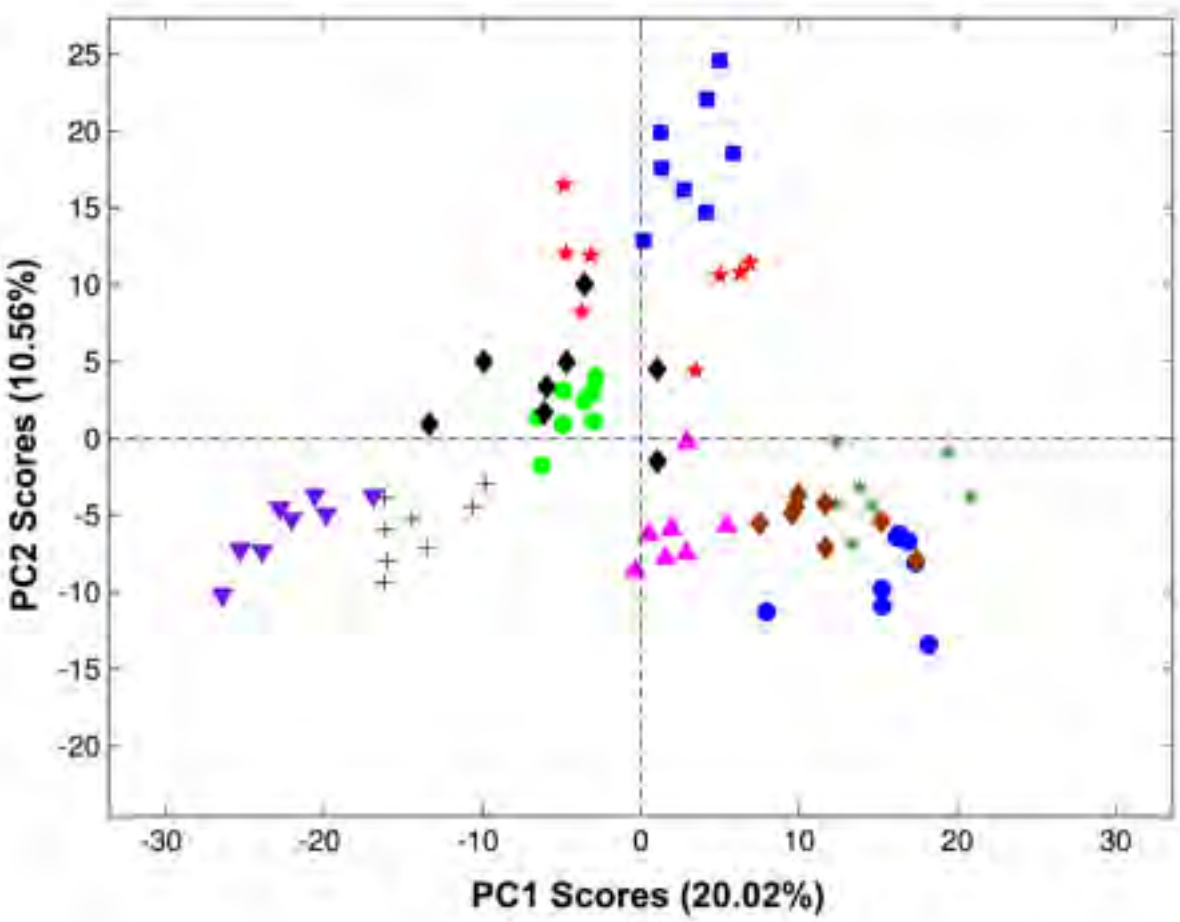


Figure 4.3 PCA analysis of FT-ICR MS data of samples of whole adult *Daphnia magna.* Biological samples of control (toxicant untreated) animals grouped based on strain; purple triangle = AW, red star = B1, blue square = B2, black cross = BD, blue circle = BK, brown diamond = EPA, green circle = IS, pink triangle = NS, black diamond = RD, green star = UPO.

In order to further investigate the basal metabolic variation between strains, PCA analysis of only untreated animals from each strain was performed (figure 4.3). Clear clustering of the samples within each strain was observed, in addition to significant clustering and separation patterns amongst the strains as a whole. The statistical difference between strains was assessed through application of ANOVA and identified that along PC1 four statistically significant groupings occur ($p = <0.05$). AW is statistically significant from all other strains as is BD which can be visualised by these samples of strains separating out from the other strains. Four strains; IS, B1, B2, RD and NS cluster tightly together along PC1 and are statistically different from the other five strains but not from each other, additional separation of these strains is observed along PC2 where B1, B2 and NS

separate out from the other groups whilst IS and RD cluster together and are not significantly different. A fourth cluster along PC1 is formed by BK, UPO and EPA which again are statistically significant from the other strains within the data set but not from each other. Therefore as a whole strains AW, BD, NS, B1 and B2 separate out individually as metabolically different strains whilst BK, EPA and UPO cluster together and are not metabolically different from each and neither are RD and IS.

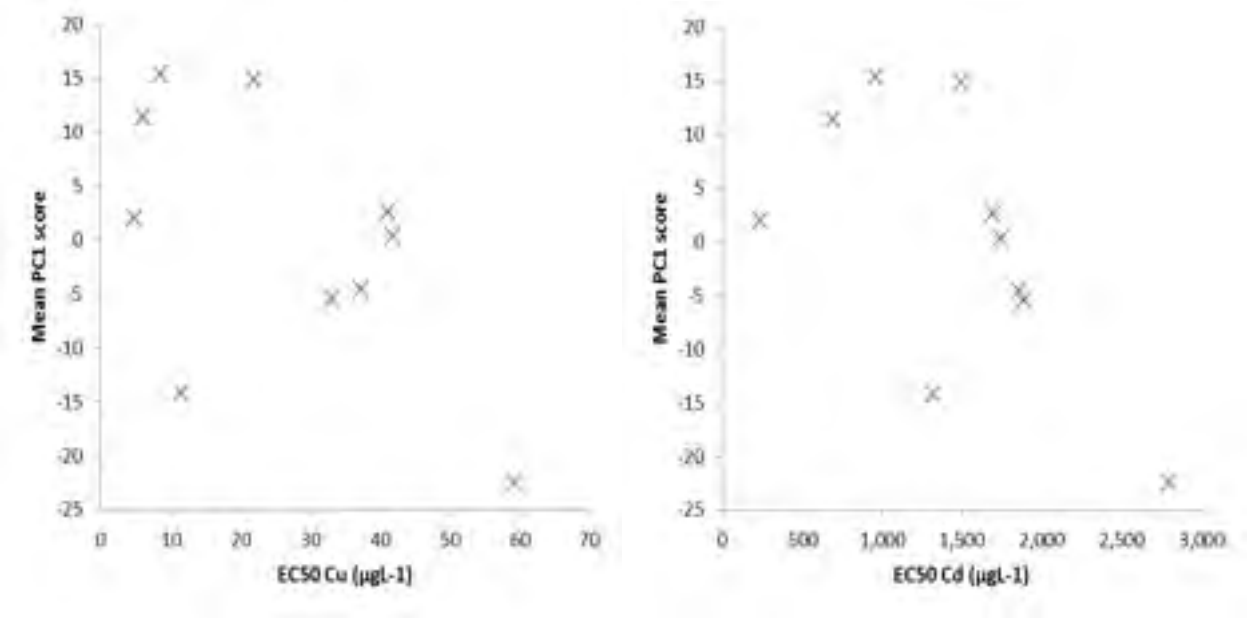
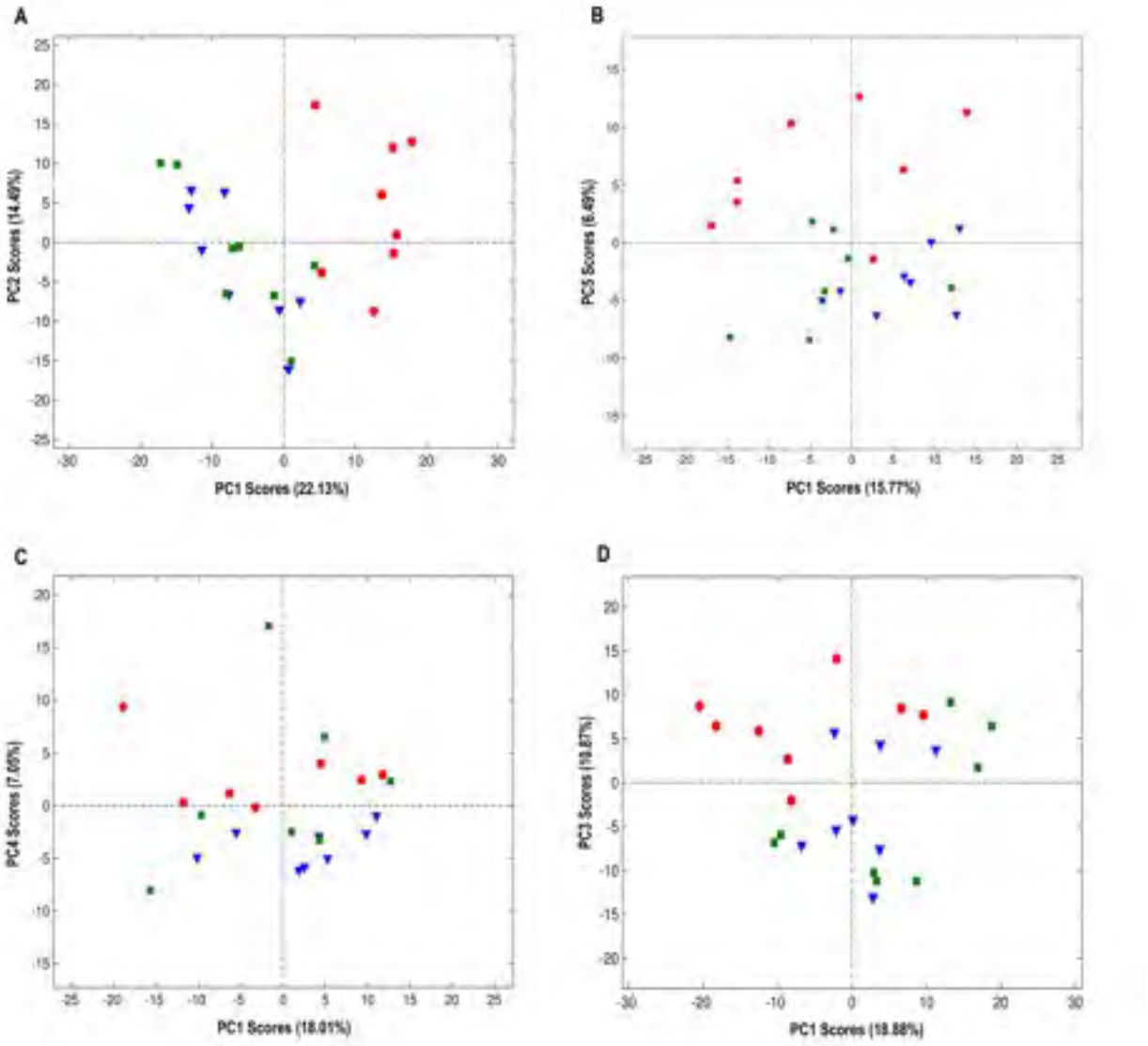


Figure 4.4 Scatter plot of mean PC1 scores against cadmium and copper EC_{50} (μgL^{-1}) across 10 different strains of *Daphnia magna*. EC_{50} calculated using TSK. Pearson's correlation between PC1 and copper, r = -0.574 (p = >0.05), and cadmium, r = -0.630 (p = <0.05).

Given that there are clear metabolic differences amongst the ten strains established in figure 4.3, the relationship between sensitivity during the acute immobilisation test (as determined in chapter 3) and basal metabolism was established (figure 4.4). Following testing for normality, the correlation between the mean PC1 scores for individual strains and EC_{50} value for that strain following exposure to cadmium or copper using Pearson's correlation coefficient (r) was established and indicates a linear dependence between the mean PC1 scores and EC_{50} following; copper, r = -0.574 (p = <0.05), and cadmium, r = -0.630 (p = <0.05) exposure. These results indicate that there is a linear relationship

between the baseline metabolism of the animals within each strain and their respective sensitivity to the heavy metals cadmium and copper.

It is clear that from the results in figure 4.2 the metabolic differences between strains dominate over treatment effects. PCA was therefore applied to each strain individually in order to investigate the metabolic variation induced by cadmium or copper treatment. Scores plots are reported in figure 4.5; from these plots a separation of cadmium treated samples from the copper and control samples across all strains is apparent. Univariate, ANOVA statistical analysis, with a Tukeys post-hoc test was applied to investigate the variance between the three treatment groups and established that across all 10 strains there was no statistical difference between the control and copper treated samples but there was a statistically significant difference between the control and cadmium treated samples within each strain.


A
PC2 Scores (14.49%)
PC1 Scores (22.13%)
B
PC3 Scores (6.49%)
PC1 Scores (15.77%)
C
PC4 Scores (7.05%)
PC1 Scores (18.01%)
D
PC3 Scores (10.87%)
PC1 Scores (18.88%)

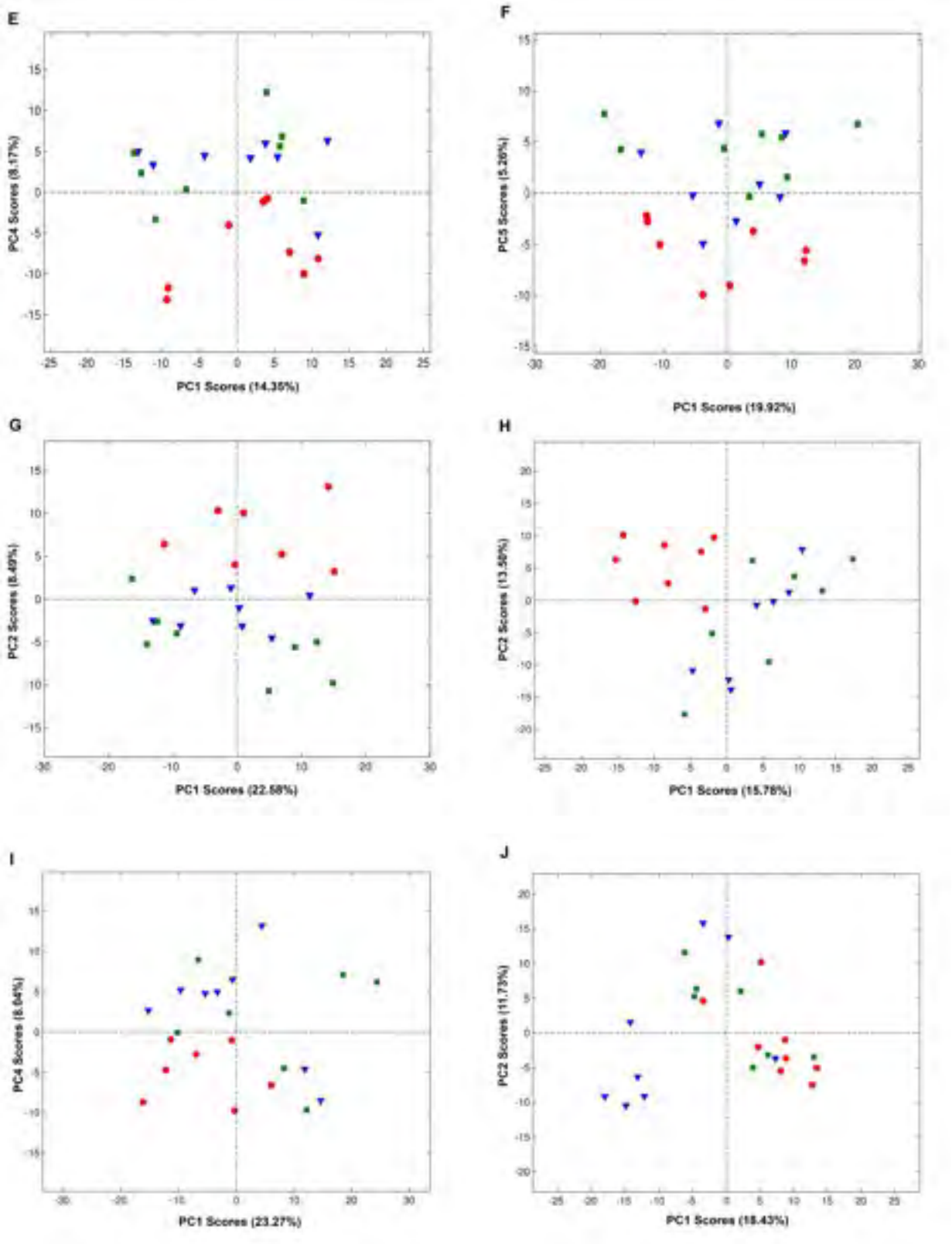


Figure 4.5 PCA analysis of FT-ICR MS data of samples of whole adult *Daphnia magna.* Biological samples grouped according to treatment; red circle = cadmium, blue triangle = control, green square = copper. (A) AW, (B) B1, (C) B2, (D) BD, (E) BK, (F) EPA, (G) IS, (H) NS, (I) RD, (J) UPO. PCs plotted as PC1 against the next most significant PC to show a significant metabolic effect following cadmium treatment.

Scores plots in figure 4.5 depict PC1 against the next lowest PC to identify a significant difference between cadmium and control samples, in the case of AW (figure 4.5 (A)) this is PC2 which accounts for 14.49% of total variance within the sample whilst for EPA (figure 4.5 (F)) PC1 was plotted against PC5, which accounts for 5.26% of the total variance.

The degree of metabolic difference following cadmium exposure was also investigated by plotting the number of significant peaks (determined through t-tests performed on normalised peak intensity values) that discriminate between control and cadmium samples within each strain and the cadmium EC_{50} value figure 4.6. No linear relationship between the degree of metabolic effect of cadmium exposure and the relative sensitivity of the each strain to cadmium was established.

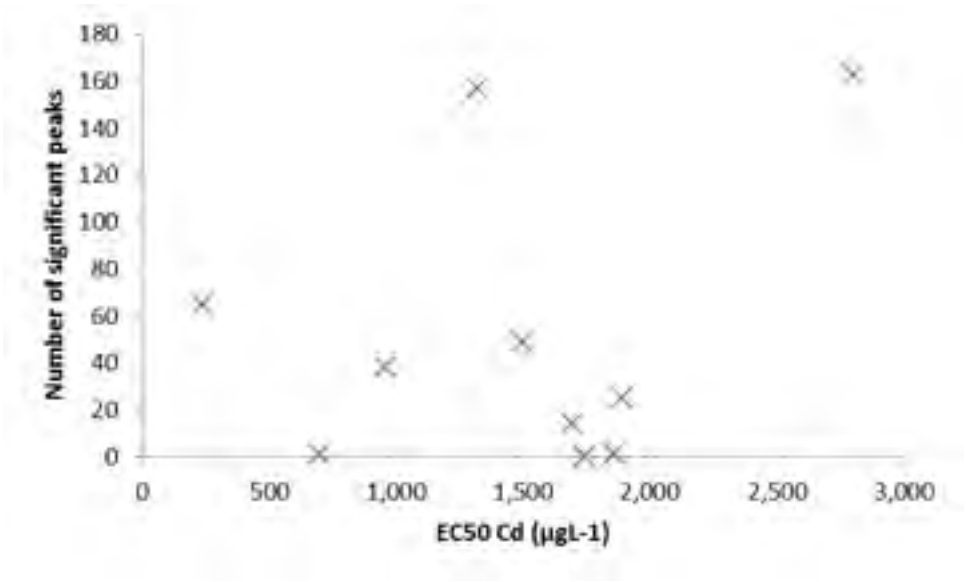


Figure 4.6 Scatter plot of number of significant peaks between control and cadmium treated samples of *Daphnia magna* against cadmium EC_{50} (μgL^{-1}) across 10 different strains. EC_{50} calculated using TSK. Statistical significance of peaks determined using student's t-test where p = <0.05

In order to further investigate the underlying metabolic effects and therefore mechanisms giving rise to the separation following cadmium treatment. Peaks within the data set that had been assigned a putative identification using MI-PACK were investigated. A student's t-test was performed to determine the peaks that are statistically significant between control and cadmium treated samples within each strain. The statistically significant peaks

that were assigned a putative ID are given in Table 4.2. The amino acids, alanine and arginine were identified, as well as three tryptophan derived alkaloids (N-Methyltryptamine, borrerine and lunamarine). With the exception of arginine, levels of these amino acids and amino acid metabolites were observed to increase, indicated by the fold changes given in table 4.2.

Simultaneously, peaks that were identified but were not statistically significant were also investigated. With specific attention paid to the amino acids, based on the fact that several were found to be significantly changing in addition to glutathione and its precursors. A large number of amino acids have been putatively identified within the data set but do not appear to be changing greatly between cadmium and control treated animals. Interestingly, a number of glutathione ion forms and pre-cursers e.g. γ-Lglutamyl-L-cysteine were identified and display an induction following cadmium treatment in several of the strains (Kidd 1997). Glutathione showed a 2.154 and 1.654 fold induction in strains AW and IS respectively following cadmium treatment, γ-Lglutamyl-L-cysteine also showed a 3.541(AW) and 2.697(UPO) fold induction (Table 4.3).

		Strain																			
		AW		B1		B2		BD		BK		EPA		IS		NS		RD		UPO	
		p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change
m/z	**ID**																				
124.01728	**Alanine**	4E-05	3.186	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
233.12535	**Arginine**	-	-	-	-	-	-	-	-	0.044	0.816	-	-	-	-	-	-	-	-	-	-
211.08173	**N-Methyltryptamine**	-	-	-	-	0.029	2.644	-	-	-	-	-	-	-	-	-	-	-	-	-	-
267.07324	**Inosine**	-	-	-	-	-	-	0.017	1.807	-	-	-	-	-	-	-	-	-	-	-	-
277.11125	**Borrerine**	-	-	-	-	0.005	2.11	-	-	-	-	-	-	0.019	2.49	-	-	4E-04	2.922	-	-
307.0856	**Lunamarine**	-	-	-	-	0.002	4.011	7E-04	2.874	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2 Table of putatively identified metabolites that change significantly between cadmium treated and control samples across 10 strains of *Daphnia magna.* Fold change between control and cadmium treated reported, p-value denotes the adjusted p-value after correction for FDR (Benjamini and Hochberg 1995).

			Strain																			
			AW		B1		B2		BD		BK		EPA		IS		NS		RD		UPO	
	Putative metabolite ID	m/z	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change
Glutathione and metabolites	S-Glutathionyl-L-cysteine	425.08025	0.321	1.358	0.61	1.265	0.32	1.939	0.428	0.704	0.619	1.183	0.736	1.301	0.938	1.151	0.862	1.052	0.561	1.57	0.229	1.287
	Glutathione	306.07642	-	2.154	-	0.848	-	1.104	-	1.010	-	1.087	-	1.097	-	1.654	-	0.991	-	0.935	-	1.164
	gamma-L-Glutamyl-L-cysteine	249.05481	0.024	3.541	0.865	1.117	0.923	1.073	0.961	0.976	0.33	1.721	0.87	0.646	0.974	1.068	0.786	0.858	0.981	0.977	0.106	2.697
Amino acids	Alanine	124.01728	4E-05	3.186	0.842	1.172	0.771	0.795	0.924	0.954	0.541	1.366	0.682	1.414	0.81	0.697	0.825	1.188	0.852	1.214	0.631	1.244
	Arginine	233.12535	0.065	0.746	0.679	0.91	0.511	0.912	0.366	0.858	0.112	1.139	0.044	0.816	0.527	0.874	0.758	1.07	0.421	0.867	0.055	0.639
	Ornithine	131.0828	0.501	1.091	0.754	0.93	0.882	1.041	0.535	0.926	0.834	1.036	0.64	0.892	0.84	0.889	0.96	1.011	0.541	0.879	0.553	1.106
	Proline	114.05622	0.804	1.114	0.701	1.11	0.991	0.993	0.892	1.042	0.493	1.136	0.789	1.163	0.836	1.144	0.944	1.032	0.514	1.108	0.102	1.354
	Serine	104.03546	0.96	1.008	0.679	0.854	0.685	0.733	0.991	0.989	0.207	1.32	0.947	1.027	0.992	1.009	0.599	1.102	0.929	1.043	0.253	1.198
	Thymine	125.03584	0.349	1.305	0.75	1.118	0.605	1.152	0.567	1.303	0.2	1.262	0.449	1.283	0.891	1.114	0.521	0.731	0.966	1.017	0.326	1.173
	Tryptophan	225.06438	0.85	0.943	0.953	0.964	0.192	1.762	0.635	1.195	0.834	0.917	0.789	1.188	0.972	1.076	0.884	0.927	0.883	1.138	0.805	0.879

Table 4.3 Table of putatively identified metabolites and their fold change between cadmium treated and control samples across 10 strains of *Daphnia magna.* Fold change between control and cadmium treated reported, p-value denotes the adjusted p-value after correction for FDR (Benjamini and Hochberg 1995). n.b. p-values are not reported for glutathione peaks as these were excluded from further data analysis during the RSD filtering process

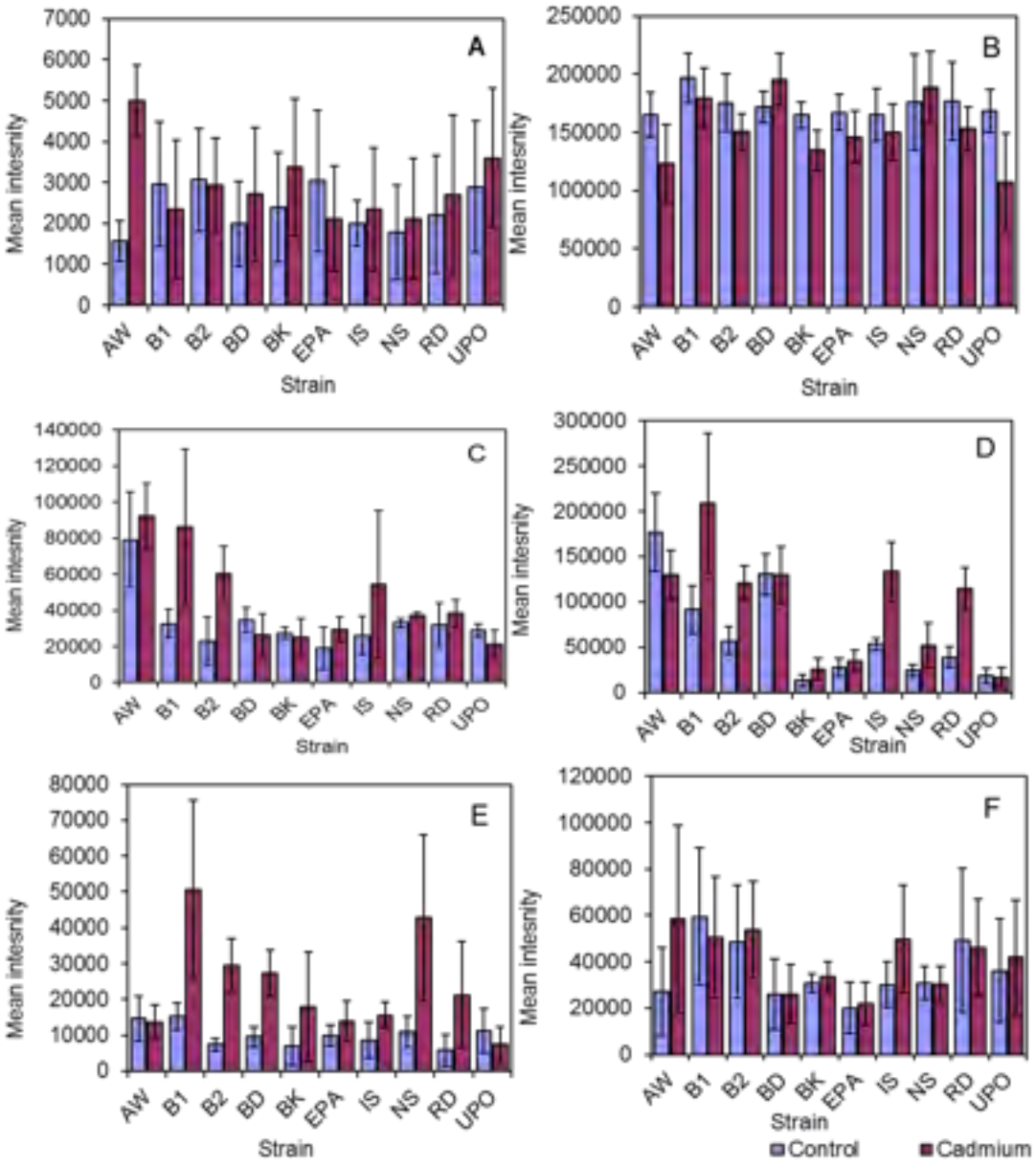


Figure 4.7 Histograms showing the mean intensity of six different peaks across 10 strains of *Daphnia magna* in cadmium and control treated samples. (A) alanine, (B) arginine, (C) N-methyltryptamine, (D) borrerine, (E) lunamarine (F) glutathione. Error bars denote standard deviation.

The intensity of significantly changing amino acid peaks and glutathione in cadmium and control samples within each strain are plotted in figure 4.7. A large fold change in alanine following cadmium exposure appears to be unique to the AW strain, interestingly the control levels of alanine within AW samples are lower than in all the other strains (figure 4.7 (A)). Although arginine was identified as being statistically significant between treatments, mean intensity values across the 10 strains appear reasonably consistent (figure 4.7(B)). Levels of lunamarine, show a large induction following cadmium treatment in a number of strains including B1, B2, BD and NS (figure 4.7 (E)). Glutathione levels show a much more varied level of intensity across the 10 strains, a relatively large fold induction is however observed following cadmium treatment in AW and IS strains, there is almost no change in intensity of glutathione peaks between treatments in the NS strain (figure 4.7 (B)).

4.4 Discussion

The data set prepared in this thesis is to date, the largest complete set of samples run within this research group. Therefore, there were significant concerns about the technical reproducibility, especially when considering the instrumental problems encountered. Results from figure 4.1 show that the reproducibility across QCs samples is very high, inferring that technical reproducibility across the entire sample set is good and the results produced are robust.

PCA models of the entire data set indentified that the *Daphnia magna* strain was the most dominant factor in discriminating between samples, irrespective of the treatment effect. This suggests not only that the 10 strains used within this thesis are indeed metabolically different but that the metabolic differences between the strains are greater than the metabolic changes induced following exposure to cadmium and copper. Together with the correlation identified between the baseline metabolism of animals within each strain and

the EC_{50} values (figure 4.4), these results suggest that phenotypic metabolic differences between each strain may be responsible for the differences in sensitivity following acute exposure of cadmium and copper observed. Genotypic differences between strains has previously been suggested to account for such variance however, genetic factors do not always manifest in downstream phenotypes depending on complex regulatory mechanisms (Picardo *et al.* 2007). Metabolism however, is a direct manifestation of the functional state of an organism, the results presented in this thesis; therefore suggest for the first time that the strains of *Daphnia magna* used here are metabolically different.

In order to investigate toxicant effects PCA models of each individual strain were created (figure 4.5), and identified significant metabolic effects following cadmium treatment but not copper. This may be due to the sub-lethal exposure level used. Copper is known to be an essential element and it is likely that at the concentration of copper used during these exposures was not toxic. Cadmium however, which is believed to be intrinsically toxic, with no beneficial role yet identified within *Daphnia magna,* did induce metabolic changes within treated animals. Based on cadmium treated samples being determined as significantly different from control animals within individual strain PCA models, attempts were made to identify the peaks and therefore metabolites responsible, to gain a mechanistic understanding. Of the total number of significant peaks between treatments across all strains only 6 peaks could be assigned a putative metabolite ID. These findings highlight the most fundamental limitation of MS-based metabolomics. The high mass accuracy of instruments gives rise to accurate peak m/z values however, there is limited information regarding specific metabolites that may give rise to these peaks.
Results in figure 4.7 suggest that amino acid metabolism appears to be affected following cadmium treatment, based on the significant fold changes in tryptophan metabolites e.g. lunamarine observed. Changes in amino acid metabolism following toxicant exposure

have been reported by a number of studies, and are generally considered to be indicative of toxicity considering the importance of amino acid homeostasis in the repair and synthesis of proteins (Jones *et al.* 2012; Dube and Hosetti 2012).

Peaks corresponding to glutathione and its precursors were also detected within the data set, from the intensity data reported in figure 4.7 clear differences in this metabolite amongst the 10 strains were observed, most notably a large fold change following cadmium treatment in the AW strain, the least sensitive strain to acute cadmium exposure. This observation raises interesting questions about whether glutathione may be important in the relative sensitivities of these strains. Those strains that show an induction may be better adapted to deal with the oxidative stress induced following heavy metal exposure through the ability to increase protective antioxidant glutathione levels. It is important to state however, that glutathione peaks were identified as being highly variable and were excluded from the data set during RSD filtering so were not used to produce final PCA models. It is possible that due to the transient nature of GSH, levels may have been affected by sample preparation processes leading to large amount of variability across the samples, which lead to a high RSD and ultimately its exclusion.

Although cadmium treatment was identified to have a significant metabolic effect, the mechanisms behind this effect are highly complex and within the limits of this project further analysis of the data beyond what is reported to investigate these mechanisms was not possible (section 6.2).

Chapter 5

Development of Targeted Molecular Assays in

Daphnia magna

5.1 Introduction

As previously discussed, exposure to cadmium and copper is associated with an induction of oxidative stress, in particular an increase in the formation of ROS and lipid peroxidation. Significant increases in ROS following sub-lethal exposure to copper has been reported in *Daphnia magna*, as well as in trout hepatocytes, where a concomitant decrease in cellular viability was observed (Manzl et al. 2004; Xie et al. 2006). Studies have also reported an increase in ROS following cadmium exposure; such effects are somewhat unexpected considering that cadmium is not redox active and therefore unable to participate in the Fenton-type chemistry through which copper gives rise to free radicals (Barata *et al.* 2005; Matović et al. 2011; Cuypers et al. 2010). It is likely therefore that cadmium induces an increase in intercellular ROS indirectly; possible mechanisms include the inhibition of protective mechanisms e.g. the suppression of ROS scavengers such as glutathione and the inhibition of antioxidant enzymes (Matović et al. 2011). Cadmium has been reported to have a high affinity for protein thiols, as the most abundant free thiol, glutathione may be an important target via this mechanism (Cuypers et al. 2010).

With a the aim of further understanding the molecular basis for cadmium and copper toxicity in addition to potentially identifying differences between strains a series of targeted assays for ROS, total protein concentration and lipid peroxidation in *Daphnia magna* were developed and discussed herein.

5.2. Development of a targeted molecular assay for the detection of Reactive Oxygen Species (ROS) in *Daphnia magna*

Historically, due to their transient nature, ROS and their induction following toxicant exposure has been studied indirectly through the activity of antioxidant enzymes including SOD and CAT (Barata *et al.* 2005). However, there is also a body of work that report

monitoring the level of ROS can be studied directly through the use of the fluorescent probe 2'7'-dichlorofluorescin diacetate (DCF-DA).

5.2.1 Detection of intercellular ROS using 2'7'-dichlorofluorescin diacetate (DCF-DA)

Early work with DCF-DA reported its use as a highly sensitive method for the detection of peroxides (Cathcart *et al.* 1983). Since then DCF-DA has been utilised successfully in the detection of ROS in both algae and trout hepatocyes (Knauert and Knauer 2008; Manzyl *et al.* 2004). More relevantly the use of DCF-DA for the detection of ROS in live and whole organism homogenates of *Daphnia magna* has also been reported (Becker *et al.* 2011; Wang *et al.* 2009; Xie *et al.* 2006, 2007). DCF-DA rapidly penetrates cellular membranes, once inside it is hydrolysed by intra-cellular esterases, to yield $DCFH_2$, a process that also contains the $DCFH_2$ within the cell (Wang *et al.* 2009). Once created $DCFH_2$ reacts rapidly with ROS to yield the fluorescent product DCF (Cathcart *et al.* 1983). The amount of fluorescence may then be quantified to indicate the amount of ROS present.

5.2.2 Materials and methods

Experiments were conducted based on a method adapted from Xie *et al.* 2006 briefly; following exposure animals were transferred to a 24-well plate (Corning®Costar®, Sigma-Aldrich, UK) in approximately 2ml of OECD media (section x.). 20µL of a 1mM DFF-DA stock in dimethyl sulphoxide (DMSO) (Invitrogen) was then added to each relevant well to a final concentration of 10mM. Following 4hr incubation in the dark with the fluorophore, fluorescence was read using a FLUOstar Omega plate reader (BMG Labtech), λ_{ex} = 485 nm λ_{em} = 520 nm. Fluorescence of DFF treated animals was reported following correction for background fluorescence of *Daphnia* in the absence of fluorophore.

Initial experiments were performed with the following modifications from the method used by Xie *et al.* 20, neonates (>24hrs old) were used in contrast to the 8 day old daphnia

reported by Xie *et al.* with the intention of making comparisons to EC_{50} experiments. In their original papers the authors used 1mM stock of DCF-DA in ethanol (EtOH), for these experiments a slightly different version of the fluorophore DFF-DA (2'7'-difluorofluorescin diacetate) in DMSO was used. In addition, in contrast to Xie *et al.* who report that they read *Daphnia* live, in these experiments *Daphnia* were immobilised immediately prior to reading the fluorescence. Following the removal of exposure media, approximately 1ml of 100% EtOH was added to each well, immobilising the daphnia rapidly (within 15 seconds) which has been shown to effectively immobilise daphnia without distorting the sample (Black and Dodsen 2003).

5.2.3 Results

5.2.3.1 Initial validation of protocol

20, <24hr neonates (n=3) were transferred to 200ml of OECD media (section 2.1) and exposed for 24hrs to a 50 μgL^{-1} dose of copper as described in section 2.3.1. *Daphnia* were transferred in exposure media to 24-well plate in exposure media and incubated with DFF-DA (total exposure time = 28 hrs).

Results shown in figure 5.1 indicate that fluorescence in the copper treated samples was lower than in control samples. This result was in contrast to the results of up to 600% increase on control fluorescence following copper treatment reported by Xie *et al.* (Xie *et al.* 2006).

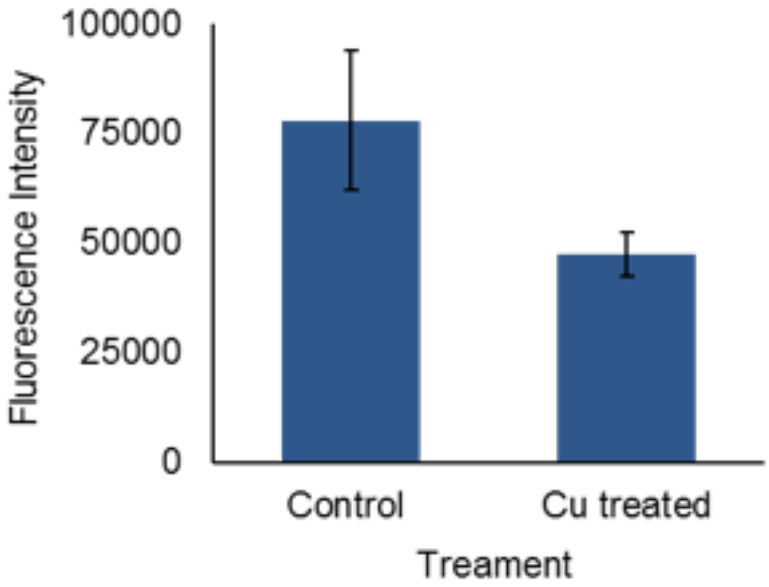


Figure 5.1 Fluorescence intensity of control and copper-treated *Daphnia magna* neonate samples incubated with 1mM DFF-DA. Fluorescence intensity reported following correction for background fluorescence. Error bars denote standard deviation, n=3.

It is proposed that the decrease in fluorescence reported here may have been due to the 28 hr exposure period relative to 4 hrs described by Xie *et al.* Following toxicant exposure it is known that protective mechanisms are mobilised in an attempt to reduce effect of ROS produced. Therefore it is possible that decrease observed is as a consequence of such protective mechanisms.

5.2.3.2. Investigation of multiple exposure concentrations and reduced exposure period

The experiment was repeated using several different dose ranges at much lower concentrations; 0, 19.7, 40, 50 and 79.4 μgL^{-1}, (n=3). Exposure period was also reduced to 4 hrs in exposure beaker followed by 4 hr incubation with DFF-DA in exposure media (8 hr total).

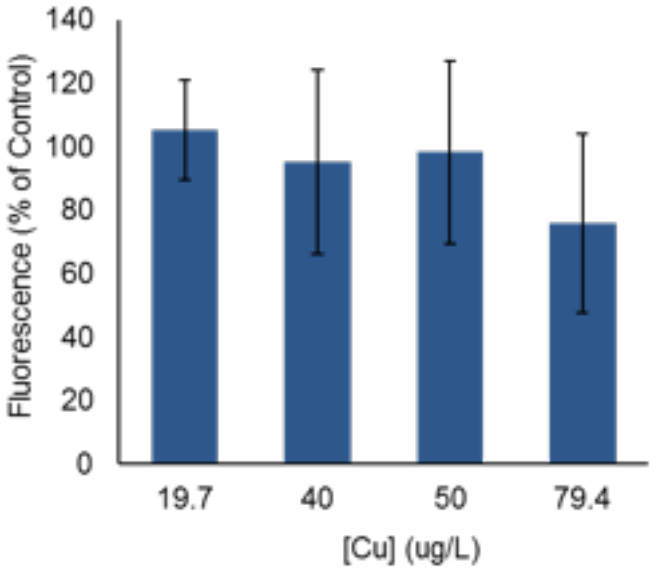


Figure 5.2 Fluorescence of *Daphnia magna* neonate samples following 8 hr exposure to different concentrations of copper. Fluorescence reported as percentage of control samples. Error bars denote standard deviation, n=3.

four concentrations, once again in contrast to the significant induction reported in the literature (Xie *et al.* 2006).In addition, poor reproducibility across the three replicates was observed, indicated by large standard deviation.

The deviation in protocol from the exact method performed by Xie *et al.* summarised in section 5.2.2 was proposed to be the source of the disparity of between our data and theirs and were addressed individually (Xie *et al.* 2006).

5.2.3.3. Validation of ethanol mediated immobilisation

In order to address the effect immobilising the animals with 100% EtOH may have on the assay, procedure in 5.2.3.2. was repeated for the 19.7 μgL^{-1} dose (n=3). Fluorescence was measured both prior to and after the addition of EtOH. Results are shown in figure 5.3.

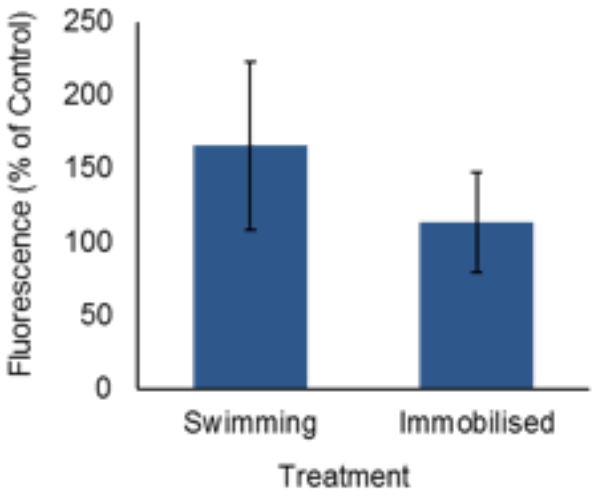
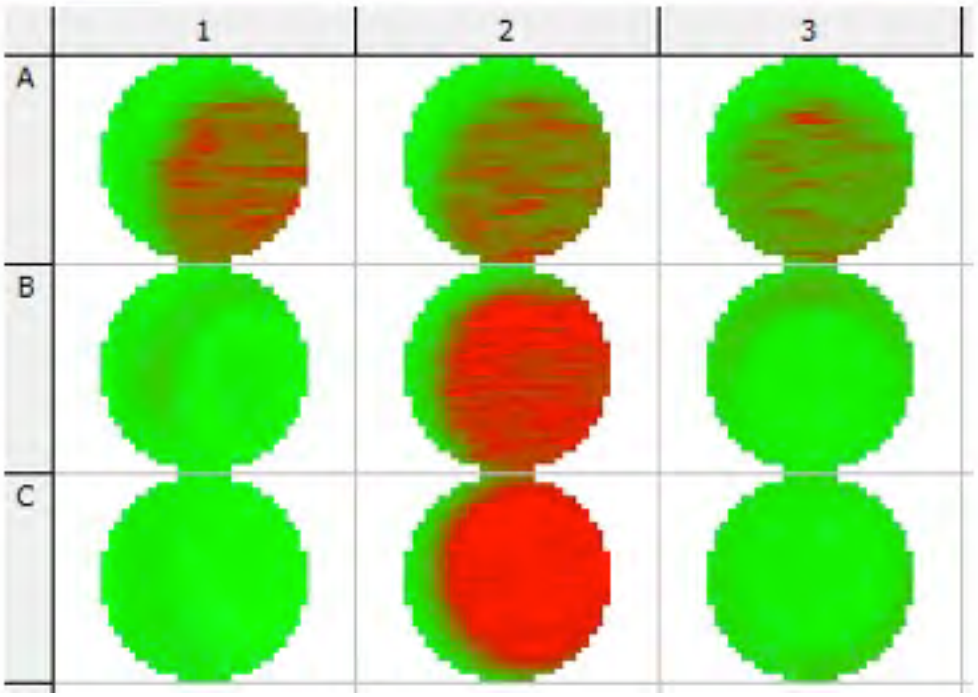


Figure 5.3 Fluorescence intensity of copper treated (19.7 µgL^{-1} dose) *Daphnia magna* neonate samples prior to and after immobilisation with 100% EtOH. Fluorescence intensity reported as percentage of control samples. Error bars denote standard deviation, n=3.

Fluorescence of each sample is considerably higher prior to immobilisation, however from the plate reader screen shots figure 5.4. it is apparent that prior to immobilisation fluorescence reported is higher because the *Daphnia* are mobile within the well and are potentially read more than once as the optic scans across the plate. In contrast, after addition of EtOH, fluorescence clearly localised around specific locations within the well, corresponding to immobilised animals. It is therefore probable that EtOH does not directly interfere with the assay, and that reading the animals prior to immobilisation produces artificially high levels of reported fluorescence as animals are read numerous times.

A



B

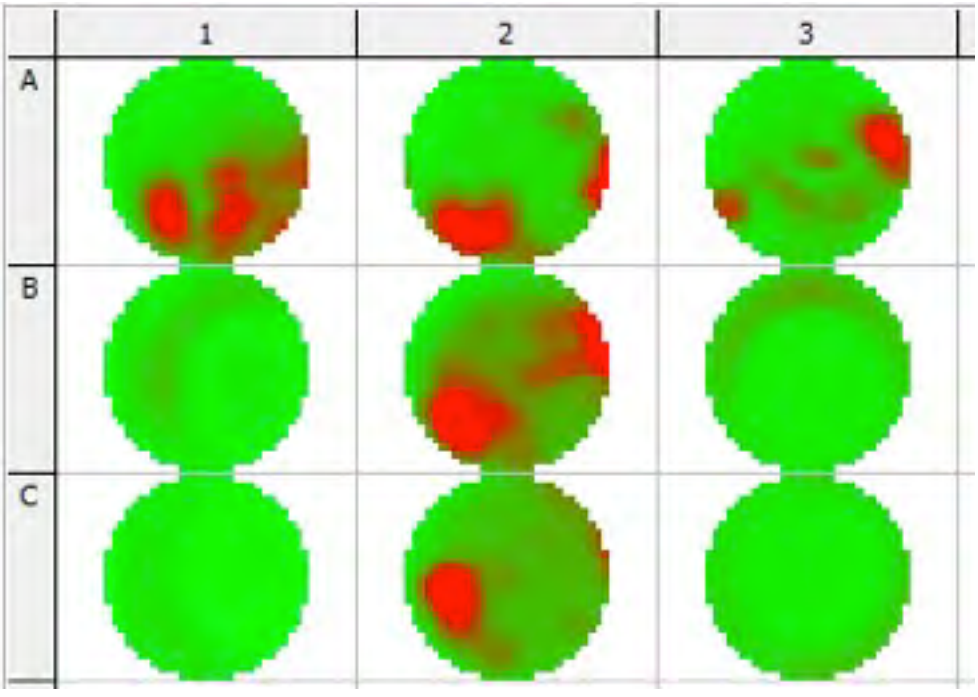


Figure 5.4 Screen shots from FLUOstar Omega microplate reader of samples of *Daphnia magna* (A) prior to immobilisation and (B) following immobilisation with 100% EOH. Red areas indicate high areas of fluorescence, green areas indicate low levels of fluorescence

5.2.3.4. Modifications to fluorophore and biomass used

The effect of the two further modifications made to the original protocol reported by Xie *et al.* were further investigated in a further experiment. The DFF-DA stock used previously had been stored for >12 months, through several freeze-thaw cycles. A new stock of DCF-DA (Invitrogen), was purchased and resuspended in EtOH (1mM stock as before). The effect of biomass was also investigated through the use of 3, 14 day old *Daphnia* in contrast to neonates used previously. *Daphnia* were exposed to copper doses of 13, 4, 1.3, 0.4 and 0.13 μgL^{-1} for a 4 hr period within the well plate in the presence of 10mM DCF-DA.

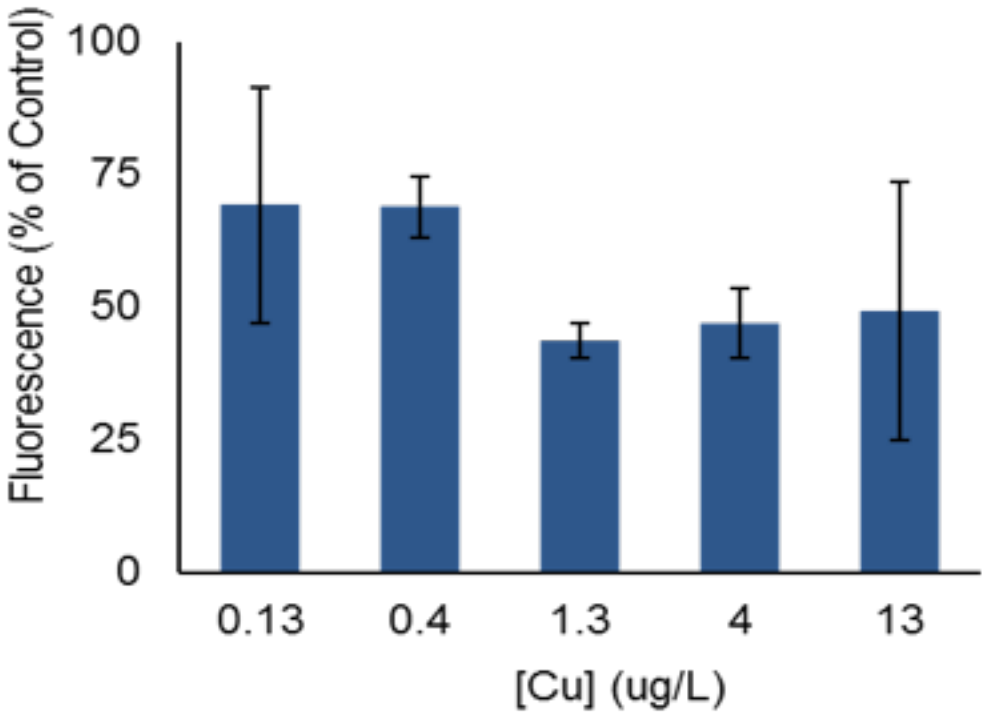


Figure 5.5 Fluorescence of *Daphnia magna* 14 day old adult samples following 4 hr exposure to different concentrations of copper. Fluorescence reported as percentage of control samples. Error bars denote standard deviation.

From the results displayed in figure 5.5 a significant decrease in fluorescence was once again observed following copper exposure, with poor reproducibility as previously reported.

5.2.3.5. Validation of technique using hydrogen peroxide and effect of biomass

At this stage a significant increase in ROS in response copper treatment across all experiments had not been observed in contrast to results reported by Xie *et al.* (Xie *et al.* 2006). In order to establish whether this was due to technical problems with the assay design or treatment related, experiments in 5.2.3.4 were validated with the use of a different toxicant: hydrogen peroxide (H_2O_2). Animals were exposed in the presence of DCF-DA for 4hrs within the well plate to concentrations: 5, 1.6, 0.5, 0.16 mM from a 49mM H_2O_2 stock (Sigma-Aldrich).

Fluorescence was divided by dry weight and expressed as a percentage of control, to observe whether differences in biomass between samples were responsible for poor reproducibility. Results reported in figure 5.6. Show that the mean fluorescence following treatment with H_2O_2 was higher than control samples across all concentrations. Indicating a potential increase in ROS. However, reproducibility across replicates was once again very poor. Correcting for dry weight did have some minor impact on this variation, particularly in the 0.16 mM treated samples. Indicating that the biomass within the sample may have considerable impact on the fluorescence observed.

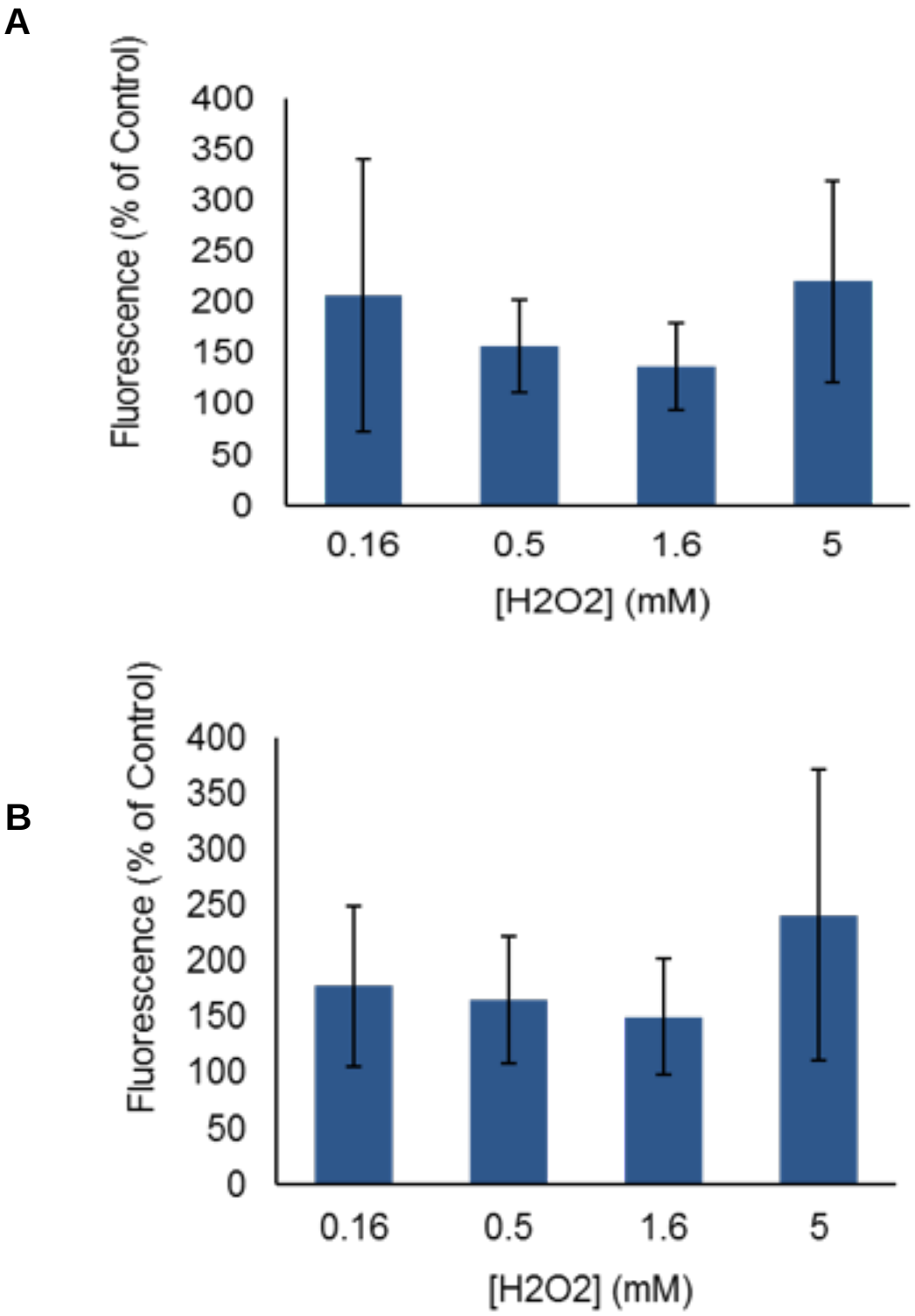


Figure 5.6 Fluorescence of *Daphnia magna* 14 day old adult samples following 4 hr exposure to different concentrations of hydrogen peroxide prior to (A) and following (B) correction for dry weight. Fluorescence reported as percentage of control samples. Error bars denote standard deviation.

5.2.4 Conclusions on the development of targeted ROS assay

Although the use of a DCF-DA based assay for the quantification of ROS has been reported successfully by several groups including its application to *Daphnia magna*, numerous difficulties were experienced here.

Notably, we were unable to reproduce the results reported previously by Xie *et al.* All reasonable efforts, within the time limitations of this project, were made to ascertain whether this was as a result of the modifications that were made to the original method. Including; the age/size of *Daphnia* used, effect of immobilisation using EtOH, potential issues with DFF-DA stock and validation of the method using a second model toxicant.

It is reasonable to suggest that the addition of EtOH immobilisation step improves the assay protocol as animals are not read multiple times during the course of fluorescence measurements.

Ultimately the significant induction of ROS following copper exposure reported by Xie *et al.* was not observed during the wide range of exposure periods and concentrations performed during this project (Xie *et al.* 2006). Due to time limitations further investigation into the reasons for this were not permitted. In addition, very poor reproducibility was observed across the biological replicates throughout all the experiments performed. The robustness of conclusions drawn from data that shows such variation is very limited. Further investigation into the reasons for this variation are warranted, in addition to validation of the exact protocol performed by Xie *et al.* that has also been applied by an independent group (Wang *et al.*2009).

5.3 Development of an assay to determine total protein content in samples of *Daphnia magna*

The amount of total protein within a biological sample is indicative of the amount of biological material present and is an important correction factor used in various biochemical assays. It may also be used to inform on a suitable amount of biomass needed to perform an assay. The development of a method to determine the total protein within samples of *Daphnia magna* is discussed in this section.

5.3.1 Application of Bio-Rad protein assay to samples of *Daphnia magna*

30 <24 hr old *Daphnia magna* neonates were collected into Precellys^{TM} homogenisation tube and flash frozen in liquid nitrogen. 250 µL of ice-cold phosphate buffered saline (PBS) (20 mM, pH 7.4) (Sigma-Aldrich, UK) was added to each tube, samples were then homogenised as described in section 2.4. The Bio-Rad protein assay (Bio-Rad Laboratories, UK), colorimetric assay based on standard Bradford protein dye-binding assay (Bradford 1976) was applied to samples. Bradford reagent was prepared by diluting concentrated stock 1:5 with dH_2O, followed by filtration through 0.4 µm filter. Standards were prepared using a 1mgml^{-1} stock of bovine serum albumin (BSA) (Sigma-Aldrich, UK); 0, 1, 2, 4, 6, 8 and 10 µL of BSA stock was added to 1ml plastic cuvettes (0, 1, 2, 4, 6, 8 and 10 µgml^{-1} final concentration). 2 µL of *Daphnia magna* homogenate was then added to sample cuvettes. Samples and standards were made up to a total 1ml using diluted Bradford reagent, stirred and incubated for 10 minutes at room temperature. Using 0 µgml^{-1} standard as a blank, absorbance for standards and samples read at 595 nm.
The absorbance of standards was used to construct a calibration curve of absorbance against known concentration of BSA, from which protein concentration of sample can be calculated (not shown).

The absorbance on *Daphnia* samples fell below the limits of detection for this assay, this was postulated to be due to the dilution factor (1:500) of samples being too high. As such there was not enough protein within the final sample to detect using this protocol

5.3.2 Application of Bio-Rad microplate assay to samples of *Daphnia magna*

The assay performed in section 5.3.1 was repeated using a smaller dilution factor within a 96-well plate (Corning®Costar®, Sigma-Aldrich, UK). A range of different samples were collected containing 30, 40, 50 and 60 <24 hr neonates (n = 3) and prepared as described in section 5.3.1.

BSA protein standards were prepared from a 100 $\mu g ml^{-1}$ stock; 0, 2, 5, 7.5, 10 and 15 µL were added to well plate in duplicate, 2 µL of prepared samples were also loaded into the well plate in duplicate. Standards and samples were made up to a total volume of 160 µL using dH_2O. 40 µL of Bio-Rad Bradford agent was then added to each well, following 5 minute incubation at room temperature absorbance at 595 nm was measured.

The absorbance of standards was used to construct a calibration curve of absorbance against known concentration of BSA (figure 5.7), from which protein concentration of sample can be calculated (figure 5.8).

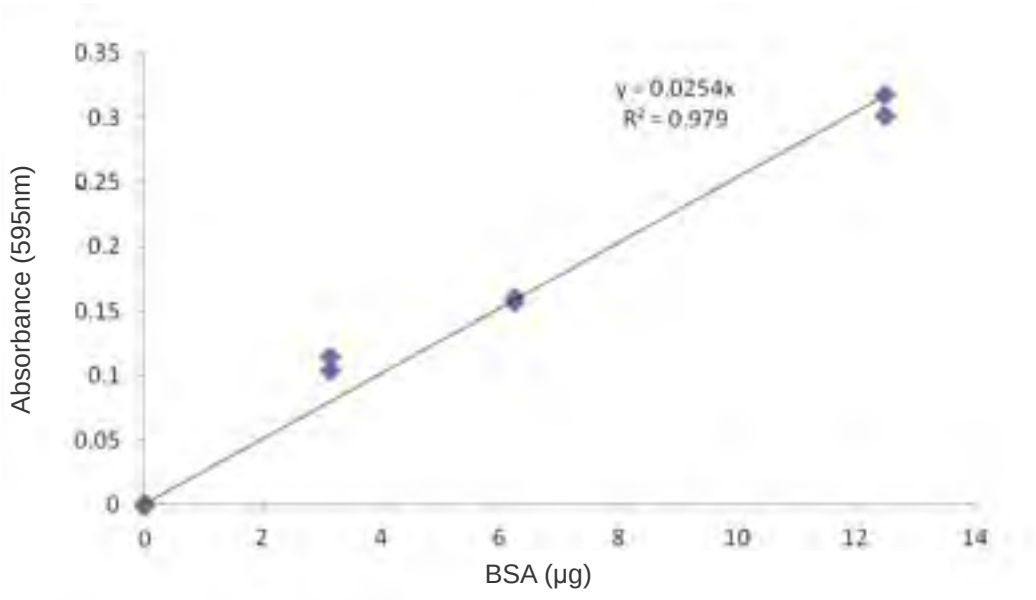


Figure 5.7 Calibration curve of absorbance (595nm) against known amounts of BSA

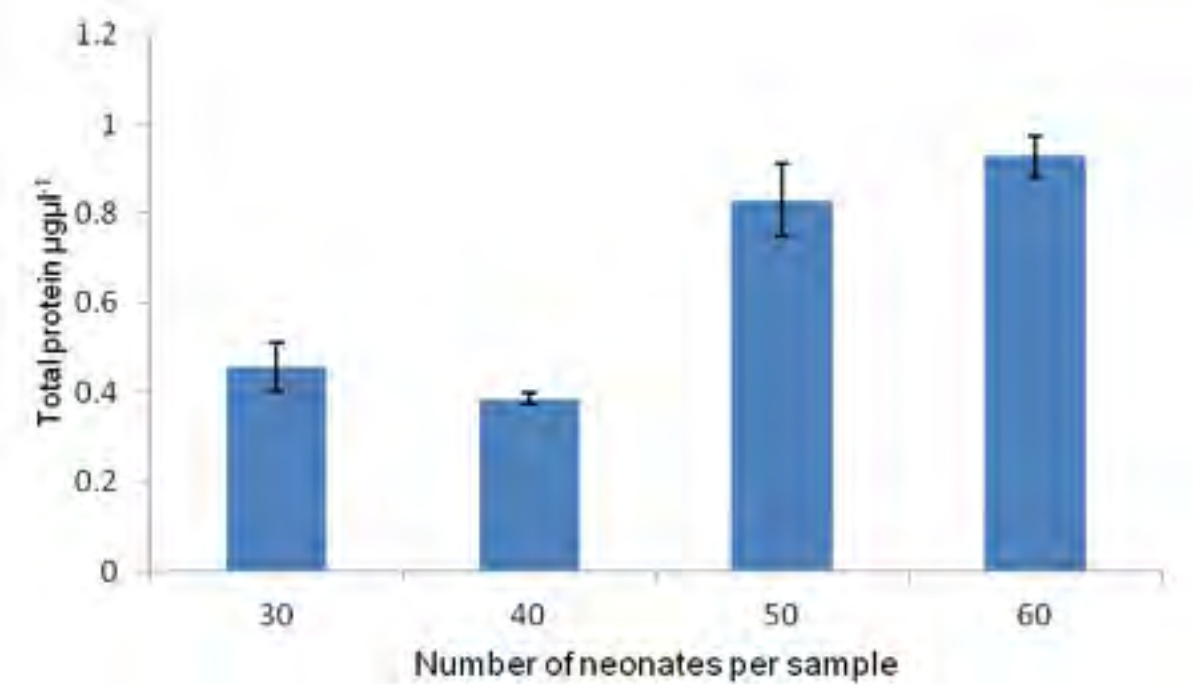


Figure 5.8 Total protein concentration of samples of different number of *Daphnia magna* neonates. Error bars denote standard deviation

The application of the Bio-Rad microplate assay to samples of *Daphnia magna* neonates proved successful, with detectable levels of protein observed. The highest total protein concentration achieved was 0.928 $\mu g \mu l^{-1}$ for samples containing 60 neonates. This concentration may however be too low for certain molecular assays and the numbers of neonates required to generate more concentrated samples were unfeasible.

5.3.3 Application of Bio-Rad DC protein assay to samples of adult *Daphnia magna*

The concentration of total protein within samples of *Daphnia* neonates was found to be too low for realistic application to molecular assays therefore the protein content of samples of 14 day old *Daphnia magna* was investigated with the use of a similar assay to that used in section 5.3.1 and 5.3.2.; the Bio-Rad DC protein assay originally adapted from the Lowry assay (Lowry *et al.* 1959). Due to concerns over the interference of cellular debris with the assay a homogenisation step was also added.

20, 14 day adults (n=4) were collected and homogenised as described previously. The homogenate was then centrifuged at 1500 rpm and 4 ^{0}C for 10 minutes and the supernatant used for protein determination. Standards were prepared from 10 mgml $^{-1}$ BSA stock and dH_2O to achieve following concentration; 0.625, 1.25, 2.5, 5 and 10 mgml $^{-1}$. 5 µL of each standard and sample was then added in duplicate to a 96-well plate. 25 µL of 'reagent A' and 200 µL of 'reagent B' added to each well and following 15 minute incubation at room temperature, absorbance at 645 nm was recorded using 0 mgml $^{-1}$ standard as a blank.

The absorbance of standards was plotted to create a standard calibration curve figure 5.9 and the concentration total protein within samples was calculated based on this curve.

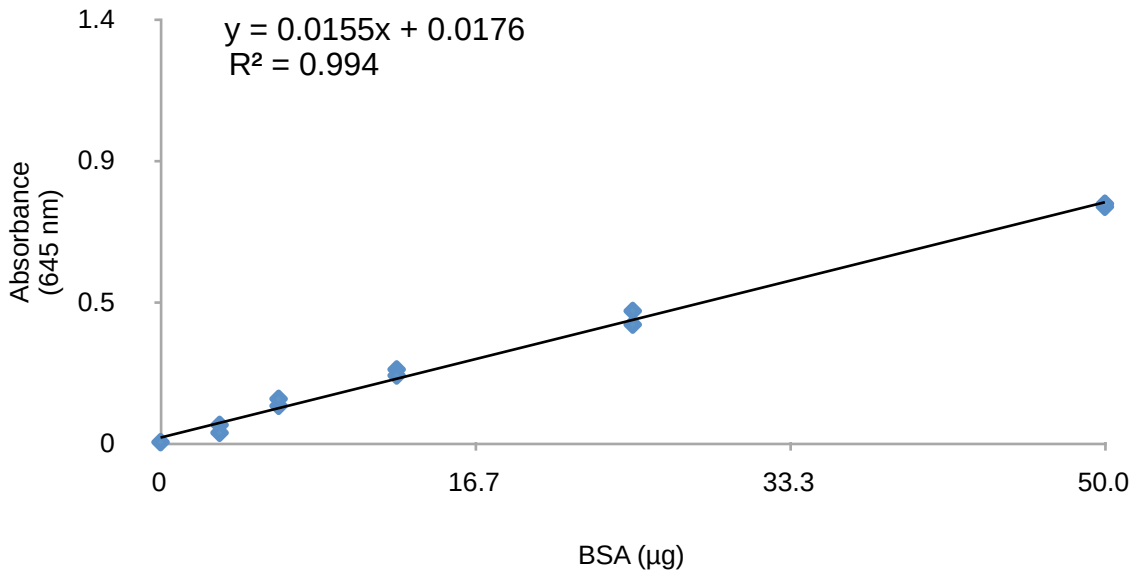


Figure 5.9 Calibration curve of absorbance (645nm) against known amounts of BSA

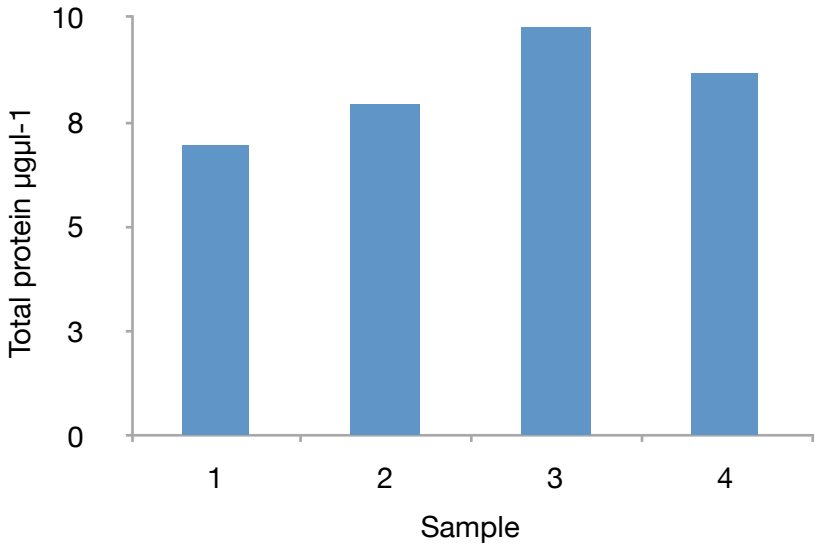


Figure 5.10 Total protein concentration of 4 samples of 20, 14 day old *Daphnia magna*.

The results in figure 5.10 show a significant increase in the concentration of protein detected using samples of 20 adult *Daphnia* was observed and falls within an appropriate range for use of samples with other targeted assays. However, the amount of protein varies considerably between samples. Therefore, it was determined that the amount of protein within each sample should be determined for use as a normalisation factor during any further targeted assays.

5.4 Application of the BIOXYTECH® LPO-586™ assay for the detection of lipid peroxidation within samples of *Daphnia magna*

Based on reports from the current literature it is has been shown that an increase in lipid peroxidation is associated with exposure to cadmium and copper.

Lipid peroxidation produces downstream products reactive aldehydes malondialdehyde (MDA) and 4-Hydoxynonenal (4-HNE) (protein-aldehyde adducts)(Houglum *et al.* 1990) Traditional experiments involve the quantification of individual downstream products of unsaturated lipid peroxides e.g. the thiobarbituric acid-reactive substances (TBARS) assay that measures the concentration of MDA. In the following experiment the presence of lipid

peroxides in *Daphnia magna* is investigated through the use of BIOXYTECH® LPO-586™ assay (*Oxis*Reasearch™) for the quantification of both MDA and 4-HNE.

In brief. 20, adult (14 day old) *Daphnia* were exposed to 50 μgL^{-1} copper for 24hrs (n=2) in 200ml of OECD media (section 2.1) in absence of food or additional supplements. Daphnia were collected and flash frozen in liquid nitrogen, 2.5 µL of 0.5M butylated hydroxytoluene (BHT) in acetonitrile was added before samples were prepared as described in section 5.3.3. BHT is an antioxidant added to prevent further lipid peroxidation occurring during sample preparation (Botsoglou *et al.* 1994). The sample supernatant was then kept on ice prior to conducting the assay according to manufactures protocol for the detection of MDA and 4-HNE (BIOXYTECH® LPO-586™ assay (*Oxis*Reasearch™) catalog number 21012).

Absorbance of assay standards and samples were read at 586 nm to yield a calibration curve (figure 5.11). The mean concentration of MDA and 4-HNE collectively within the samples can then be calculated based on this standard curve and are reported in figure 5.11.

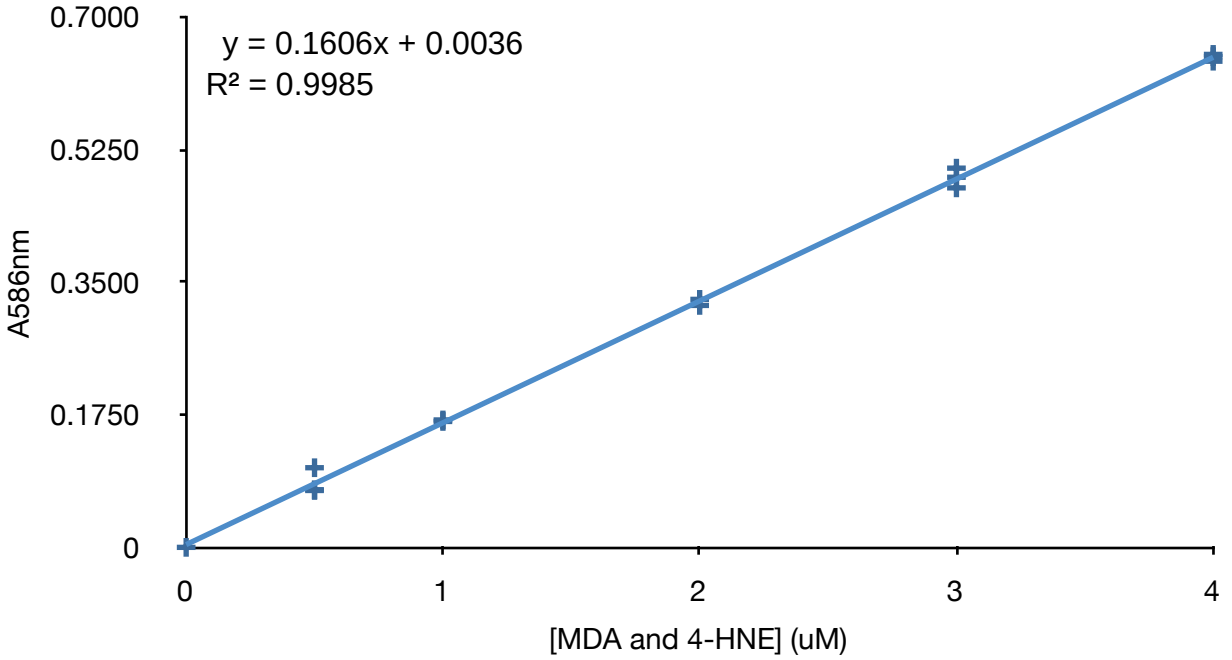


Figure 5.11 Calibration curve of absorbance (586nm) against known amounts of lipid peroxide standard.

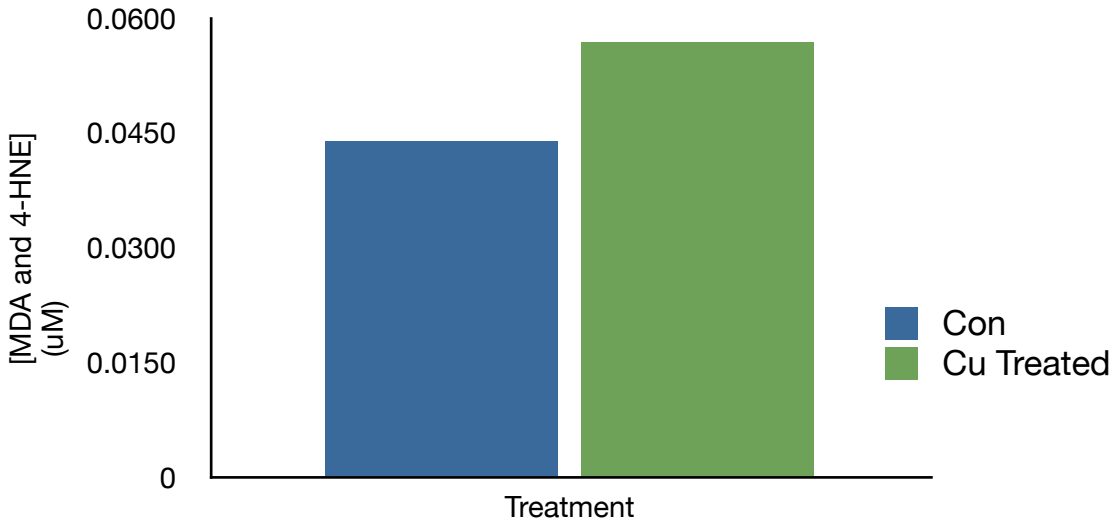


Figure 5.12 Total lipid peroxide concentration of samples of 20, 14 day old *Daphnia magna*. Blue = control sample, Green = following 24 hr exposure to 50 μgL^{-1} copper. Error bars denote standard deviation.

Results in figue 5.12 show that the concentration of MDA and 4-HNE within the samples fell within the detectable range of this assay, however similar to the protein assay described in section 5.3, the original assay was performed in 1ml cuvettes following a substantial sample dilution. Considering the protein assay has been successfully adapted for use in a well plate, the same may be possible with here. This would considerably reduce the amount of reagents required and therefore cost of performing the assay, in addition, to increasing the number of technical replicates that can be obtained from the same sample as a smaller volume is required. From the results in figure 5.12 it does appear that some lipid peroxidation is increased in the copper treated animals but the standard deviation represented by the error bars is high and is derived from only three replicates. Unfortunately due to time limitations, further investigation and development of this assay was not possible. However the successful application of this assay kit and subsequent detection of lipid peroxides within *Daphnia magna* samples makes this a promising avenue of investigation with regards to further elucidating a mechanistic basis behind the strain differences in sensitivity to cadmium and copper observed in chapters 3 and 4.

Chapter 6

Final Conclusions and Further Work

6.1 Conclusion

The main aim of this thesis was to assess strain to strain differences in *Daphnia magna* following exposure to the heavy metals cadmium and copper. Three individual aims were stated in section 1. 10 and were addressed separately within the chapters of this thesis. The first of these aims was to address whether there are any differences in response between strains of *daphnia magna* following acute exposure to cadmium and copper. Results in Chapter 3 established that there are marked differences in the response of the 10 strains of *Daphnia magna* to acute copper and cadmium exposure assessed using the acute immobilisation test, confirming previous reports within the scientific literature as discussed in section 1.6. *Daphnia magna* is a model organism used extensively throughout ecotoxicological testing, the results of which are used to inform on regulatory decision making. The differences in sensitivity between strains identified in this thesis represent an important limitation of the data obtained from such tests in that, results from independent laboratories using different strains of *Daphnia* may not agree, consequently having major repercussions on toxicity classification of substances in regulatory toxicology. The second aim of this thesis was, for the first time, to apply MS-based metabolomics to different strains of *Daphnia magna* to investigate if there are metabolic differences between strains, in addition to addressing the metabolic effects of sub-lethal exposure to cadmium and copper. The results presented in Chapter 4 showed that there are significant differences in baseline metabolism within the 10 strains of *Daphnia magna* investigated, and that the baseline metabolism of these strains correlated with their relative sensitivity to cadmium and copper. This observation suggests a plausible mechanistic basis for the differences in sensitivity to acute exposure identified in Chapter 3.

Together these results suggest there is an urgent need to account for the variation between strains of *Daphnia magna* in ecotoxicological testing. This could be achieved

through the validation of results by an independent laboratory however; a more robust method would be to maintain several independent cultures within the same laboratory, potentially including an internationally standardised strain of known sensitivity.

The elucidation of cadmium and copper induced metabolic changes within Chapter 3 proved more complex. No significant metabolic differences were observed between control and copper treated samples, potentially due to the dose level used within the exposures being below a toxic level. Metabolic differences between cadmium treated and control samples were observed, and results appear to suggest that amino acid metabolism and glutathione levels may be effected. Ultimately further elucidation of the metabolic changes induced following cadmium treatment were hindered by an inability to identify the metabolites concerned, which remains the fundamental limitation of MS-based metabolomics studies.

The third and final aim of this thesis was to develop a series of targeted molecular assays for use with *Daphnia magna* with a view to investigating the mechanisms behind cadmium and copper toxicity. The progress made in Chapter 5 has lead to the development of an optimised assay for the determination of total protein in *Daphnia magna*. Lipid peroxides where successfully detected within *Daphnia magna* although there is potential for further optimisation of this procedure. Efforts to detect ROS within *Daphnia magna* were met with considerable challenges, notably the poor reproducibility of this assay. Whilst significant progress was made with method development, further optimisation of both the lipid peroxidation and ROS assays is needed before their successful application; such work was not possible within the time limits of this thesis.

6.2 Further work

Following from the results presented in this thesis further work should firstly involve repeating the exposures carried out in Chapter 3 following a longer period of acclimatisation e.g. 6 months to ensure that the differences in sensitivity observed were not artefacts of strains still acclimatising to a change in culture conditions.

The results presented in Chapter 4 identified large differences in metabolism between the 10 strains; these differences were notably larger than the toxicant induced metabolic changes. Here a single exposure dose was applied across all strains. The application of different doses relative to the sensitivity of the strain e.g. 10% of the individual EC_{50} would in theory produce a standardised toxic response, and may facilitate further investigation into the mechanistic effects of cadmium and copper toxicity.

Further development of targeted assays discussed in Chapter 5 is also needed including the optimisation of the protocols and additional investigation with both cadmium and copper treatments that could not be completed within this thesis.

Chapter 7

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Chapter 8

Appendix

A. External ion calibrant list

Empirical formula	Ion form	Theoretical exact mass (Da)
CH_2O_2	$[M+^{35}Cl]^-$	80.97488
$C_3H_7NO_2$	$[M-H]^-$	88.04040
$C_3H_6O_3$	$[M-H]^-$	89.02442
CH_2O_2	$[M+Ac]^-$	105.01933
$C_2H_4O_2$	$[M+Ac]^-$	119.03498
$C_5H_{10}N_2O_3$	$[M-H]^-$	145.06187
$C_5H_9NO_4$	$[M-H]^-$	146.04588
$C_6H_{12}O_6$	$[M-H]^-$	179.05611
$C_{11}H_{12}N_2O_2$	$[M-H]^-$	203.08260
$C_6H_{12}O_6$	$[M+Ac]^-$	239.07724
$C_{16}H_{32}O_2$	$[M-H]^-$	255.23295
$C_{10}H_{13}N_5O_4$	$[M+^{35}Cl]^-$	302.06616
$C_8H_{20}NO_6P$	$[M+Ac]^-$	316.11668
$C_{18}H_{35}NO$	$[M+^{35}Cl]^-$	316.24127
$C_{18}H_{35}NO$	$[M+^{37}Cl]^-$	318.23832
$C_{18}H_{35}NO$	$[M+Ac]^-$	340.28572
$C_{22}H_{43}NO$	$[M+^{35}Cl]^-$	372.30387
$C_{22}H_{43}NO$	$[M+^{37}Cl]^-$	374.30092
$C_{22}H_{43}NO$	$[M+Ac]^-$	396.34832
$C_{22}H_{26}O_6$	$[M+^{35}Cl]^-$	421.14234
$C_{22}H_{26}O_6$	$[M+^{37}Cl]^-$	423.13939
$C_{22}H_{26}O_6$	$[M+Ac]^-$	445.18679

B. Final MS plate order

Dark grey shaded cells indicate samples that failed due to technical problems

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	**st QC**				**QC 1**				**QC 2**				**QC 3**				blank 1				UPO con 1			
B	UPO Cu 1				UPO Cd 1				RD con 1				RD Cu1				RD Cd1				AZ con 1			
C	**QC 4**				AZ Cu 1				AZ Cd1				NS con1				NS Cu1				NS Cd1			
D	EPA Con 1				EPA Cu1				**QC 5**				EPA Cd1				BK con1				BK Cu1			
E	BK Cd1				AW con 1				AW Cu 1				AW Cd1				**QC 6**				B1 con 1			
F	B1 Cu1				B1 Cd1				B2 con 10				B2 Cu 1				B2 Cd1				BD con1			
G	**QC 7**				BD Cu1				BD Cd1				EPA con 2				EPA Cu 2				EPA Cd2			
H	BD con2				BD Cu 2				**QC 8**				BD Cd2				AZ con 2				AZ Cu9			
I	AZ Cd2				NS con 2				NS Cu 2				NS Cd 9				**QC 9**				B2 con 2			
J	B2 Cu2				B2 Cd2				RD con 2				RD Cu 2				RD Cd2				AW con 2			
K	**QC 10**				AW Cu9				AW Cd9				B1 con 2				B1 Cu2				B1 Cd 2			
L	UPO con 2				UPO Cu9				**QC 11**				UPO Cd2				BK Cu2				BK Con2			
M	BK Cd2				B2 con3				B2 Cu3				B2 Cd3				**QC 12**				BK con3			
N	BK Cu3				BK Cd3				AW con 3				AW Cu3				AW Cd3				NS con3			
O	**QC 13**				NS Cu3				NS Cd3				UPO con3				UPO Cu 3				UPO Cd3			
P	BD con 3				BD Cu3				**QC 14**				BD Cd3				AZ con 3				**QC 15**			

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	**QC05**				blank_1				UPO con1				UPO Cu1				UPO Cd1				RD con1			
B	RD Cu1				RD Cd1				AZ con1				**QC06**				AZ Cu1				AZ Cd1			
C	NS con1				NS Cu1				NS Cd1				EPA con1				EPA Cu1				**QC07**			
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	**QC 08**				AZ Cu3				AZ Cd3				B1 con3				B1 Cu3				B1 Cd3			
B	EPA con3				EPA Cu3				**QC 09**				EPA Cd3				RD con3				RD Cu3			
C	RD Cd3				EPA con4				EPA Cu4				EPA Cd4				**QC 10**				BK con4			
D	BK Cu4				BK Cd4				AW con4				AW Cu4				AW Cd4				B1 con4			
E	**QC 11**				B1 Cu4				B1 Cd4				UPO con4				UPO Cu4				UPO Cd4			
F	RD con4				RD Cu9				**QC 12**				RD Cd4				AZ con4				AZ Cu4			
G	AZ Cd4				NS con4				NS Cu4				NS Cd10				**QC 13**				B2 con4			
H	B2 Cu9				B2 Cd4				BD con4				BD Cu4				BD Cd4				UPO con5			
I	**QC 14**				blank 2				UPO Cu5				UPO Cd5				RD con5				RD Cu5			
J	RDCd5				AZ con5				AZ Cu5				**QC 15**				AZ Cd5				NS Con5			
K	NS Cu5				NS Cd5				EPA Con5				EPA Cu5				EPA Cd5				**QC 16**			
L	BK Con5				BK Cu5				BK Cd5				AW Con5				AW Cu5				AW Cd5			
M	B1 Con5				**QC 17**				B1Cu5				B1 Cd5				B2 Con5				B2 Cu5			
N	B2 Cd5				BD Con5				BD Cu5				**QC 18**				BD Cd5				EPA con6			
O	EPA Cu6				EPA Cd6				BD con6				BD Cu6				BD Cd6				**QC 19**			
P	AZ con6				AZ Cu6				AZ Cd6				NS con6				NS Cu6				**QC 20**			

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	**QC21**				EPA Cd1				BK con1				BK Cu1				BK Cd1				AW con1			
B	AW Cu1				AW Cd1				**QC22**				B1 con1				B1 Cu1				B1 Cd1			
C	B2 con10				B2 cu1				B2 Cd1				BD con1				**QC23**				BD Cu1			
D	BD Cd1				EPA con2				EPA Cu2				EPA Cd2				BD con2				BD Cu2			
E	**QC24**				BD Cd2				AZ con2				AZ Cu9				AZ Cd2				NS con2			
F	NS Cu2				NS Cd9				**QC25**				B2 con2				B2 Cu2				B2 Cd2			
G	RD con2				RD Cu2				RD Cd2				AW con2				**QC26**				AW Cu9			
H	AW Cd9				B1 con2				B1 Cu2				B1 Cd2				UPO con2				UPO Cu9			
I	**QC27**				UPO Cd2				BK Cu2				BK con2				BK Cd2				B2 con3			
J	B2 Cu3				B2 Cd3				**QC28**				blank3				NS Cd6				B2 con6			
K	B2 Cu6				B2 Cd6				RD con6				RD Cu6				RD Cd6				**QC29**			
L	AW con6				AW Cu6				AW Cd6				B1 con6				B1 Cu6				B1 Cd6			
M	UPO Con6				**QC30**				UPO Cu6				UPO Cd6				BK con6				BK Cu6			
N	BK Cd6				B2 con7				B2 Cu7				**QC31**				B2 Cd7				BK con7			
O	**BK Cu7**				BK Cd7				AW con7				AW Cu7				AW Cd7				**QC32**			
P	NS Con7				NS Cu7				NS Cd7				UPO con7				UPO Cu7				**QC33**			

Plate 5

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	**QC38**				NScon4				NSCu4				NSCd10				B2con4				B2Cu9			
B	B2Cd4				BDcon4				BDCu4				BDCd4				UPOcon5				UPOCu5			
C	UPOCd5				**QC39**				RDcon5				RDCu5				RDCd5				AZcon5			
D	AZCu5				AZCd5				NSCon5				NSCu5				NSCd5				EPACon5			
E	EPACu5				EPACd5				**QC40**				BKCon5				BKCu5				UPOCd7			
F	BDcon7				BDCu7				BDCd7				AZcon7				AZCu7				AZCd7			
G	B1con7				B1Cu7				B1Cd7				**QC41**				EPAcon7				EPACu7			
H	EPACd9				RDcon7				RDCu7				RDCd7				EPAcon9				EPACu8			
I	EPACd10				BKcon8				BKCu8				BKCd8				**QC42**				AWcon8			
J	AWCu8				AWCd8				B1con8				B1Cu8				B1Cd8				UPOcon8			
K	UPOCu9				UPOCd8				RDcon8				RDCu8				RDCd8				**QC43**			
L	AZcon8				AZCu8				AZCd8				NScon8				NSCu8				NSCd8			
M	B2con9				B2Cu8				B2Cd8				BDcon8				BDCu8				BDCd8			
N	blank4				**QC44**																			
O																								
P																								

Plate 6

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	**QC34**				EPAcon4				EPACu4				EPACd4				BKcon4				BKCu4			
B	BKCd4				AWcon4				**QC35**				AWCu4				AWCd4				B1con4			
C	B1Cu4				B1Cd4				UPOcon4				UPOCu4				**QC36**				UPOCd4			
D	RDcon4				RDCu9				RDCd4				AZcon4				AZCu4				AZCd4			
E	Blank 4				**QC37**																			
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								