Proteomic approach to oxidative stress in *Daphnia magna*

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I, Louis-Charles Rainville, certify that this thesis is my own work and I have not obtained a degree in this university or elsewhere on the basis of the work submitted in this thesis.

Louis-Charles Rainville

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Abstract

The keystone aquatic organism *Daphnia magna* is extensively used to assess the toxicity of chemicals. This has recently lead to an increase in the omics literature focusing on daphnids, an increase fuelled by the sequencing of the *Daphnia pulex* genome. Yet, no omics study has looked directly at oxidative stress (OS) in daphnids, even though OS is of primary importance in the response of aquatic organisms to their changing environment and is often induced by anthropogenic xenobiotics. This thesis thus focuses on the application of redox-proteomics, the study of the oxidative modification of proteins, to *D. magna*. Specifically, daphnids were exposed to copper or paraquat, two well studied prooxidants, and protein carbonyls were labelled with fluorescein-5-thiosemicarbazide prior to two-dimensional electrophoresis (2DE). This showed clearly that both compounds affect a different portion of the proteome. The identified proteins indicated that energy metabolism was affected by paraquat, while copper induced a reduction of the heat shock response (heat shock proteins, proteases and chaperones) a counterintuitive result which may be adaptative to metal toxicity in arthropods.

The same approach was then applied to the study of the toxicity mechanism of silver nanoparticles (AgNP), an increasingly utilised form of silver with expected environmental toxicity, and its comparison to silver nitrate. The results demonstrate that, although less toxic than silver ions, AgNP toxicity functions through a different mechanism. AgNP toxicity is thus not a product of silver dissolution and increased protein carbonylation indicates that AgNP cause OS. Interestingly three of the four tested compounds altered vitellogenin levels and oxidation. Vitellogenins could thus represent an interesting subproteome for the detection of stress in daphnids. Finally, an experiment with oxidised BSA demonstrates the applicability of solid phase hydrazide in the enrichment of undigested carbonylated proteins.

Abbreviations

1DE	One-dimensional electrophoresis
2DE	Two-dimensional electrophoresis
AgNP	Silver nanoparticles
ANOVA	Analysis of variance
BODIPY	Boron-dipyrromethene
BSA	Bovine serum albumin
CI	Confidence interval
DNPH	Dinitrophenylhydrazine
EC	Environment Canada
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FTC	Fluorescein-5-thiosemicarbazide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione transferase
HNE	4-hydroxynonenal
HP	Hypothetical protein
HSP	Heat-shock protein
HSR	Heat-shock response
IAF	5-iodoacetamidofluorescein
IPG	Immobilised pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
MALDI	Matrix assisted laser desorption/ionisation
MCO	Metal-catalysed oxidation
MoA	Mode of action
MS	Mass spectrometry
NCBI	National center for biotechnology information
NP	Nanoparticle
OECD	Organisation for economic co-operation and development
OS	Oxidative stress
PES	Protein expression signature
PMSF	Phenylmethylsulfonyl fluoride
PTM	Post-translational modifications
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAgNP	Supernatant of the silver nanoparticles suspension
SOD	Superoxide dismutase
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SRM	Specific reaction monitoring
SPH	Solid-phase hydrazide
TCA	Trichloroacetic acid
TEF	Translational elongation factor
TOF	Time of flight mass spectrometry
USEPA	United-States environmental protection agency
vtg	vitellogenin

Chapter 1

Introduction

1.1 Ecotoxicology

Although man impacted on his environment from early times, the rise of industry following the two world wars led to a sharp increase of the number and impact of man-made products on the environment. It is now estimated that there are over 140 000 chemicals on the market (Massey and Jacobs, 2013), chemicals that will inevitably reach the environment. They are released accidentally, through normal use or as an inescapable consequence of industrialisation. In response to the observation of the adverse effects of pollutants on ecosystems, the field of ecotoxicology emerged over the second half of the 20th century. Broadly speaking, "ecotoxicology aims at characterizing, understanding and predicting deleterious effects of chemicals on biological systems" (Schüürmann and Markert, 1998). In order to do so, ecotoxicology must be multidisiplinary, relying mostly, but not exclusively, on the fields of chemistry, ecology and toxicology. This is required as the impact of pollutants begins at the molecular level and trickles up to the ecosystem, affecting all levels of biological organisation (Fig. 1.1). Ecotoxicology thus attempts to study the relationship between ecosytems, chemical pollutants and living organisms (Dowling and Sheehan, 2006). One of the main challenges

lies in the fact that those same chemicals which are now known to impact negatively on the environment are also responsible for the massive increase in quality of life that characterised the 20th century. Economic and social development are main drivers of human progress and consequently of chemical industries. Ecotoxicology is therefore constantly faced with the challenge of emerging categories of pollutants and the often opposite needs of society and the environment. One must also add, as pollutants are products of human economies, that ecotoxicologists are sometimes required to advise government agencies in determining policies concerning chemical pollution. Thus, the relationship between ecotoxicology and policy makers cannot be overlooked. There is a requirement for ecotoxicology to help to develop predictors of toxicity for environmental samples, industrial effluents or newly developed compounds. Also, new tools are required to identify principal causes of observed environmental impacts, in a manner analogous to human toxicology.

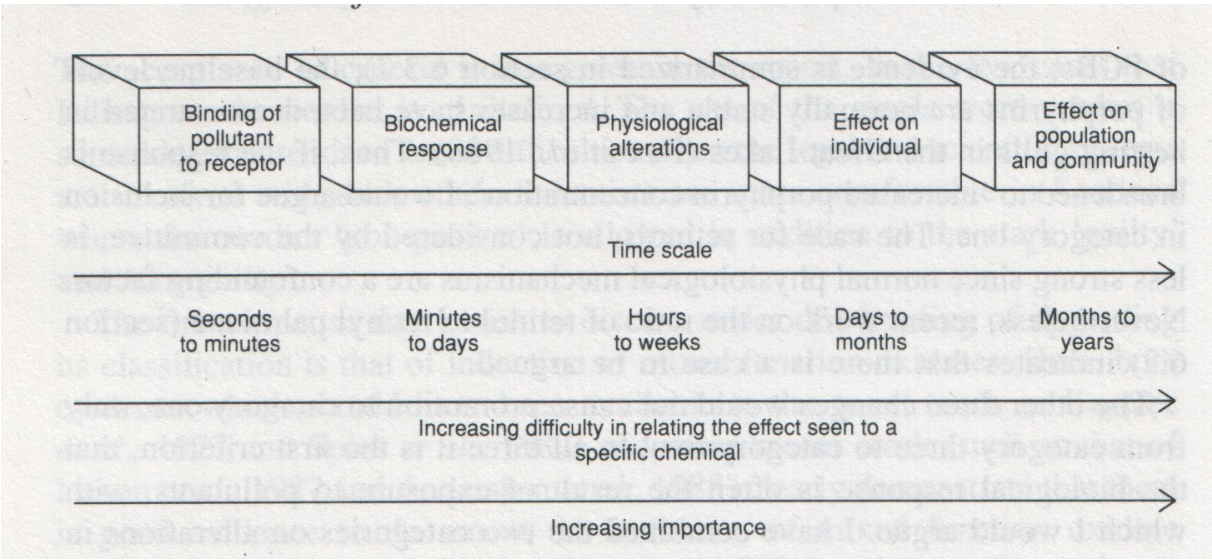

Figure 1.1: Linkages between biochemical, physiological, individual and population responses to pollutants (taken from Peakall, 1992).

In order to achieve this, ecotoxicologists often use sentinel species and molecular biomarkers. Sentinel species are organisms that are used to infer the health of an ecosystem, either because of their higher sensitivity to stressors or through

their particular characteristics (*e.g.*: sessile, non-migratory, filter-feeders, hyper-accumulators) (Wetzel, 1998). They are expected to indicate the presence of stress on an ecosystem before irreversible damage is done. Biomarkers fulfil a similar role but at the molecular or cellular level, enabling us to detect pollutant impacts before they are visible at the organism or population level (Livingstone and Goldfarb, 1998). They can be used either in the field, being then measured in sentinel species, or in the laboratory, to study potential impacts of pollutants. Such laboratory experiments on effluents or new compounds are used to assess their toxicity and also for regulatory purposes.

1.2 Daphnids

It is of course impossible to fully study the impact of all xenobiotics, or even just one xenobiotic, on all the elements of an ecosystem, nor on all the ecosystems present on Earth (Jørgensen, 1998). It is therefore necessary to limit the range of species tested in the field (sentinels), or in the laboratory, in order for ecotoxicological studies to be possible at all. The choice of species must be well considered, both in terms of the ecological importance of the test organism as well as on a practical level.

Practical limitations are particularly important for laboratory experiments, where the maintenance of healthy cultures of the organism quickly becomes resource and labour intensive (Persoone and Janssen, 1993). For these reasons, relatively few organisms are routinely used in the laboratory for toxicological studies. In the case of freshwater environments, one of the most studied taxa, if not the most studied, are members of the Daphniidae family.

Daphnids are planktonic crustaceans that are ubiquitous in freshwater environments worldwide. They are filter feeders, feeding on suspended particles and microorganisms, mainly planktonic algae (Ebert, 2005). Their role as pelagic

grazers makes them an essential part of the food chain in lakes, often as a primary food source for fish (Shaw et al., 2008). Predation is thus a major determinant of species distribution, the larger species being found mostly in predator-free environments (Ebert, 2005).

Their important ecological role along with their wide geographical distribution has made them a widely studied family and one of the most used organisms for laboratory environmental toxicity evaluation of both isolated compounds and effluents. This is in addition to their cyclical parthenogenetic reproduction (Fig. 1.2), which enables maintenance of clonal lines in the laboratory. This reduces biological variability, as well as opening the possibility of using a particular clonal line in different laboratories. These lines are also relatively simple to maintain in the laboratory.

Their ecological relevance and relative ease of culture has lead to the use of daphnids for regulatory purposes in many countries worldwide (Persoone and Janssen, 1993). This is in addition to their short life span, which enables chronic toxicity reproduction tests as well as acute toxicity testing. Their importance in the field of ecotoxicology is emphasised by the national and international guidelines offering standardised protocols for toxicity testing on daphnids, mainly *Daphnia magna* (EC, 2000; OECD, 2008, 2004), *Ceriodaphnia dubia* (EC, 2007; USEPA, 2002) and *Daphnia pulex* (OECD, 2004).There is thus an extensive database of acute and chronic toxicities in daphnids, both for pure compounds and for environmentally relevant mixtures. Despite this extensive litterature, there are surprisingly few biomolecular studies on daphnids. This is now changing though, as daphnias were the first crustaceans to have their genome sequenced (starting with *D. pulex* (Colbourne et al., 2011), there is also an ongoing project to sequence the genome of *D. magna* - https://daphnia.cgb.indiana.edu).

In addition to the large amount of laboratory data on daphnids, their ecology has also been widely studied over the last century (Shaw et al., 2008) and they

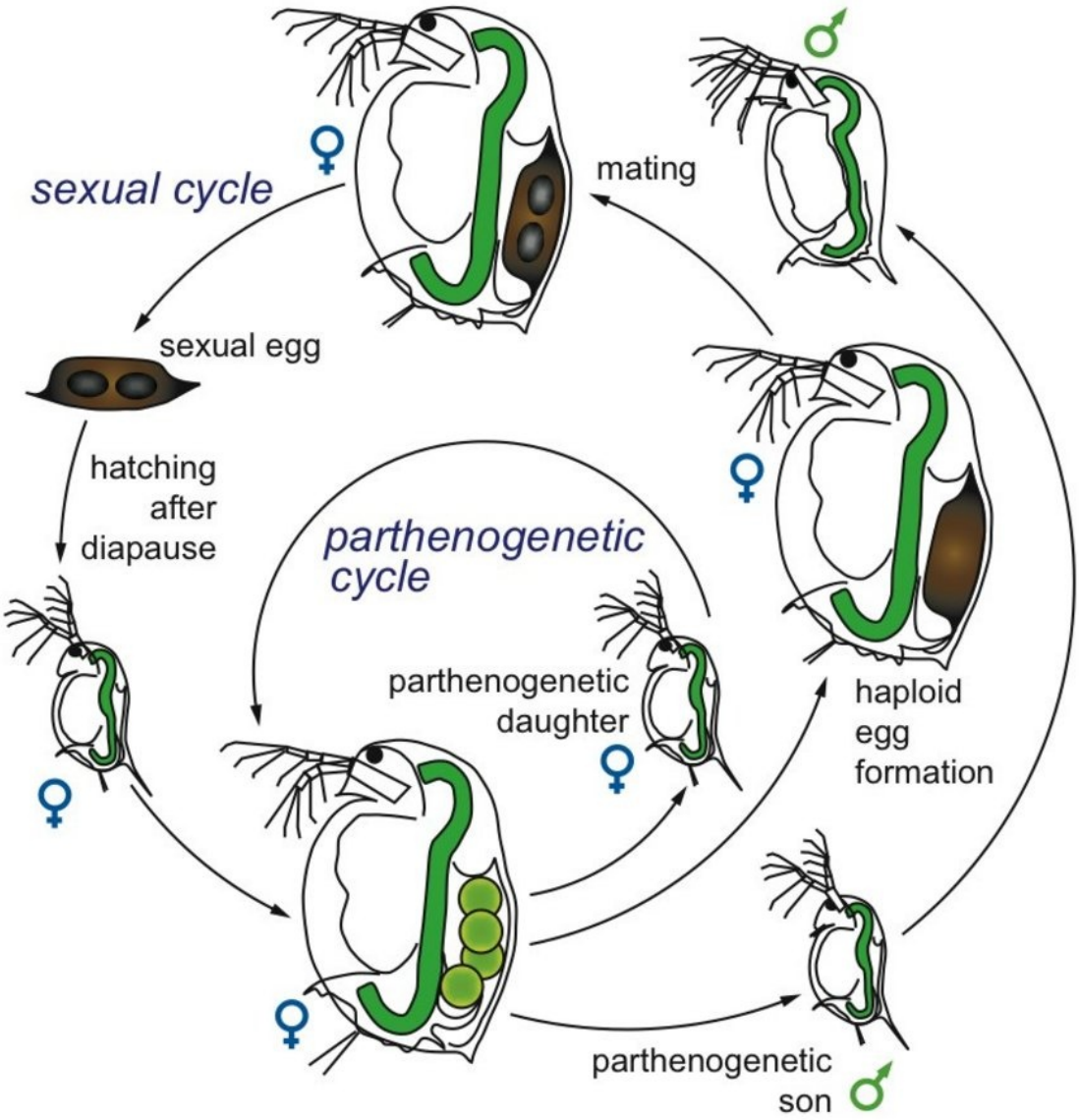

Figure 1.2: Depending on environmental conditions, female daphnias will produce diploid eggs, which will develop into clonal males or females, or produce haploid eggs, leading to sexual reproduction and formation of an ephipia that will rest in the sediments for extended periods of time before hatching (taken from Ebert, 2005).

are thus a prime candidate to link the impact of biochemical modifications to their ecosystemic effects. As mentioned above, there has been relatively limited biomolecular studies involving this taxa, especially when compared to fish and mussels. This can be understood because of practical difficulties in obtaining sufficient material either from the field or in the laboratory. Nonetheless, the amount of ecological information available on daphnids is unique and makes them a particularly interesting model for molecular studies. As molecular markers are becoming ever more prevalent in ecotoxicology, both for modelling and regulatory purposes, daphnids can be expected to receive increased attention.

1.3 Biomarkers

As with laboratory model organisms such as rodents, a better understanding of the mechanism of actions of toxicants on sentinel species can help predict and detect environmental toxicity. Such an understanding can only come from detailed studies of the biochemical response of organisms to various toxicants, and are impossible on a large scale. Yet, it is possible to link whole-organism end points to biomarker responses, which are potentially more sensitive (Lam, 2009). Thus biomarkers could help determine the presence of toxic stress before the appearance of organism- or population-level effects.

Biomarkers can be broadly defined as "xenobiotically induced variations in cellular or biochemical components, processes, structures or functions" (Wetzel, 1998). This covers many different types of measurements, including enzymatic activities, protein or mRNA expression levels, changes in organelle properties (*e.g.* Neutral red retention; Lowe et al., 1995) and measurement of immune functions (Croxton et al., 2012). Despite many promises, and the popularity of certain markers, reports of biomarkers specific to a xenobiotic or a class of xenobiotics are still relatively rare (Lam, 2009), with the notable exception of acetylcholine

esterase activities for organophosphates (Peakall, 1992) and aminolevulinic acid dehydratase for lead (Lam, 2009). This problem is not unique to ecotoxicology, as similar issues have arisen in the biomedical field (Schiess, 2008). There is thus a need for further research in order to identify biomarkers specific to certain toxicants or mechanisms of action, as well as to validate new potential biomarkers in the laboratory and in the field.

The direction of biomarker research in ecotoxicology has tended to borrow strongly from various other fields of science. New methods developed for the biomedical and molecular biology fields are now widely used by ecotoxicologists, and the systems biology approach is one of the most promising for the future. "Systems biology studies biological systems by systematically perturbing them (biologically, genetically, or chemically); monitoring the gene, protein, and informational pathway responses; integrating these data; and ultimately, formulating mathematical models that describe the structure of the system and its response to individual perturbations" (Ideker et al., 2001).

As modelling is now such a prevalent tool in ecotoxicology (Devillers, 2009), mostly concerned with the chemical and/or ecological elements of ecotoxicology, systems biology offers the promise of integrating suborganism responses into modelling. There is, of course, much research required but, as the Human Genome Project was a driving factor behind the emergence of systems biology, the Daphnia Genomics Consortium (http://wfleabase.org/) opens the door to the application of a systems biology approach using daphnids as a model organism. This is illustrated by the Computational Toxicology project (http://epa.gov/comptox) initiated by the United-States Environmental Protection Agency (USEPA) in order "to better understand the relationships between sources of environmental pollutant exposure and adverse outcomes." Within this initiative, the USEPA plans to use dapnids as a model organism in the Daphnia pilot project (http://www.epa.gov/heasd/edrb/comptox.html) to use omics

in order to link exposure to observed effects at the animal level. As the ecology of daphnids has been well studied, this raises the possibility of linking biochemical endpoints with community level effects.

The development of biomolecular, discovery based, approaches in daphnids is one of the emerging phenomenona in ecotoxicology. Biochemical data was surprisingly rare for daphnids only 15 years ago, when compared to other environmentally relevant models such as mussels and fish and to the amount of data available for the taxa (Shaw et al., 2008). However there has now been an explosion over the last years in the application of omics approaches in daphnids, with the recent publication of many studies using transcriptomics (Heckman et al., 2006; Shaw et al., 2007; Poynton et al., 2008a,b, 2011, 2012; Jansen et al., 2013), proteomics (Le et al., 2013; Fröhlich et al., 2009; Schwerin et al., 2009; Zeis et al., 2009, 2013) and metabolomics (Bunescu et al., 2010; Taylor et al., 2009, 2010; Nagato et al., 2013). This is in addition to, and in close relation with, the recent sequencing of the *Daphnia pulex* genome (Colbourne et al., 2011) and the current project to sequence *Daphnia magna* (https://wiki.cgb.indiana.edu/display/DGC/Daphnia+magna+Genome).

This rapid change in the studies involving daphnids is driven by the development of new computational and laboratory methods. These open the door for the modelling of the impact of toxicants in a manner that is based on the impact of xenobiotics on subcellular pathways and which could be linked to organism-level responses, thus predicting better the impact of known contamination and emerging contaminants. For this goal to be achieved, there is still a need to develop and apply molecular biology methods to daphnids. In this domain, the best-studied species is *D. magna*.

In this context, the present thesis focuses on the application of proteomics, specifically redox-proteomics, to the keystone species *D. magna*, and its application to the study of emerging contaminants such as silver nanoparticles (AgNP).

1.4 Proteomics

Traditionally, the use of biomarkers relies on prior knowledge of toxicity targets or of the biochemical response of organisms. This limits the use of biomarkers to the study of known contaminants and their effects or to the measurement of a non-specific stress response shared across a wide range of organisms (Thompson et al., 2012). This hypothesis-driven approach now coexists with the more recent discovery-based approaches, made available by the methodological developments of the last decades. These methods, widely known as omics in biology, aim at defining the elements of a system in order to create a database (Ideker et al., 2001). Omics will not replace traditional biomarkers but offer new opportunities in ecotoxicology, and the potential to integrate the developing field of systems biology into ecotoxicological studies.

A major impact of the development of omics is the widening of the the concept of biomarker to include expression signatures, where the expression of many proteins (*i.e.* protein expression signatures – PES) or genes are used as a multi-component signature of exposure to chemical or other stress (Vioque-Fernández et al., 2009; Bradley, 2012). A signature based on a concept similar to the one underlying the measurement of species composition, where the presence and abundance of not only single species is considered but also changes of species assemblage (Johnston and Roberts, 2009). As omics approaches gather suborganismic information, they are also widely expected to lead to the discovery of novel contaminant specific "traditional" biomarkers.

Obtaining meaningful suborganism information from proteomics experiments requires identification of the proteins forming the PES associated with a treatment. Protein identification requires the availability of mRNA, gene or protein sequences, or *ab initio* sequencing of the proteins of interest, a laborious and expensive process. This is an issue in ecotoxicology as there is very little sequence information available for most organisms of interest (Monsinjon and Knigge, 2007).

As the genome of *D. pulex* is now available, the identification of proteins from daphnids, and potentially crustaceans, is greatly simplified, genomic information is also useful for species related to the one which is sequenced (Fröhlich et al., 2009). This is why this project focuses on the use of proteomics in *D. magna*, as a high rate of identification can be expected for proteins of interest.

Proteomics studies the entire complement of proteins expressed spatially and temporally in an organism, organ or tissue, including protein variants and post-translational modifications (PTM), and the characterisation of protein-protein interactions (Martyniuk and Denslow, 2009). The entire complement of proteins in a given sample form its proteome, and proteomics approaches aim at covering as much of the proteome in a single measurement as possible. This is not achievable in practice, as proteins present very diverse physico-chemical properties as well as a range of concentrations spanning well over five orders of magnitude (Corthals et al., 2000). No method can analyse the whole dynamic range of proteins in a cell, tissue or organism, the method chosen will thus determine the results obtained to some extent. This has led some authors to reintroduce, at least in part, an hypothesis-driven approach in proteomics (Lay et al., 2006). This is done by chosing a subproteome to analyse, as it is possible to study specifically a subclass of proteins (*e.g.*: membrane proteins, affinity-selected proteins, PTM) (Osburn et al., 2011; Medvedev et al., 2012), thus reducing the complexity of the sample while increasing the sensitivity of the analysis, if the subproteome is well chosen. This maintains the advantage of a discovery-based approach: the proteins to be detected are unknown at the start of the analysis, only the subproteome is targeted. Yet it is possible to complement hypothesis-free approaches with specific reaction monitoring (SRM), a targeted mass spectrometry technique (Picotti and Aebersold, 2012). This is of particular interest in "non-traditional" species such as daphnids, as typical in-depth analysis of a toxicant's mode of action (MoA) relies on the study of specific proteins by Western blot,

and thus on specific antibodies. These are usually not available for proteins from ecotoxicologically-relevant species, and it can be hoped that SRM will replace, at least in part, Western blotting for this purpose, in addition to circumventing many of the technical pitfalls associated with antibody-based measurements (Aebersold et al., 2013).

Redox-proteomics is one of the approaches reintroducing a prior hypothesis in the proteomics field: The treatment of interest alters specifically the redox status of proteins. The different PTM associated to the redox status of cells and proteins enable the definition of different subproteomes, which are now known to vary as a result of toxicity mechanisms leading to oxidative stress (OS).

1.5 Oxidative stress and Redox-proteomics

Oxidative stress results from an imbalance in cells between reactive oxygen and nitrogen species (ROS and RNS) and their scavenging mechanisms (Fig. 1.3) (Valavanidis et al., 2006). As normal aerobic metabolism generates ROS, early cells evolved molecular and enzymatic defences in order to maintain a balance between production and scavenging of reactive species, thus maintaining the redox-homeostasis of cells. There is also a large amount of data demonstrating the importance of redox processes for signal transduction in healthy cells (Jones, 2008).

In healthy cells, most ROS react quickly with antioxidant molecules and enzymes, before it is possible for them to cause irreversible damage to DNA, lipids or proteins. Yet this balance is fragile and when more ROS and RNS are produced than the cell can buffer, a state of OS is entered. Oxidative stress is linked to ageing (Stadtman, 2006), diseases (Sultana et al., 2010), toxic stress (Valavanidis et al., 2006) and environmental factors (Borgeraas and Hessen, 2002). Having often been related to pollutants, its interest in ecotoxicology is now undeniable

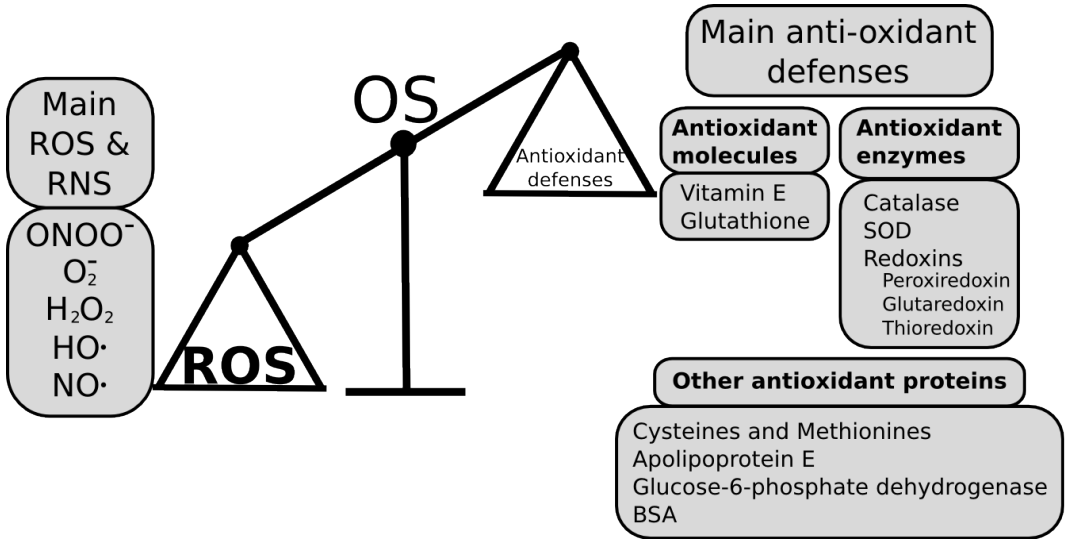

Figure 1.3: Maintenance of cellular homeostasis requires a balance between the formation of oxidative and nitrosative species and the anti-oxidant defenses of the cells. Taken from Sheehan et al. (2012)

and measures of OS are widespread. These include measurements of antioxidant enzymes and molecules (Regoli et al., 2002) but also of damage caused by ROS, of which lipid peroxidation (Marnett, 2002) and DNA damage (Shugart, 2000) are the most frequently measured in ecotoxicology. Yet, proteins are expected to absorb and sustain most of the ROS-induced damage in cells, up to 70 % (Davies, 2005). Many "lesions" are formed when ROS and RNS react with proteins. The most frequently studied, protein carbonyls and thiol oxidations, are illustrated in Figure 1.4.

Redox-proteomics tools are available to study the most common oxidative lesions of proteins: carbonyls, cysteine (thiol) oxidation, tyrosine nitrosylation and methionine oxidation (Sheehan et al., 2012). To this day, oxidative lesions of proteins have received little attention in ecotoxicology. Of the above lesions, protein carbonylation gathered the most attention in ecotoxicological studies (Braconi et al., 2011). Although most only measured carbonylation levels as a sign of OS (Almroth et al., 2005; Tedesco et al., 2008; Atamaniuk et al., 2013) some did apply a redox-proteomics perspective. These studies have focused on oxidation of thiols in mussels (McDonagh and Sheehan, 2007; Tedesco et al., 2010b,a, 2012)

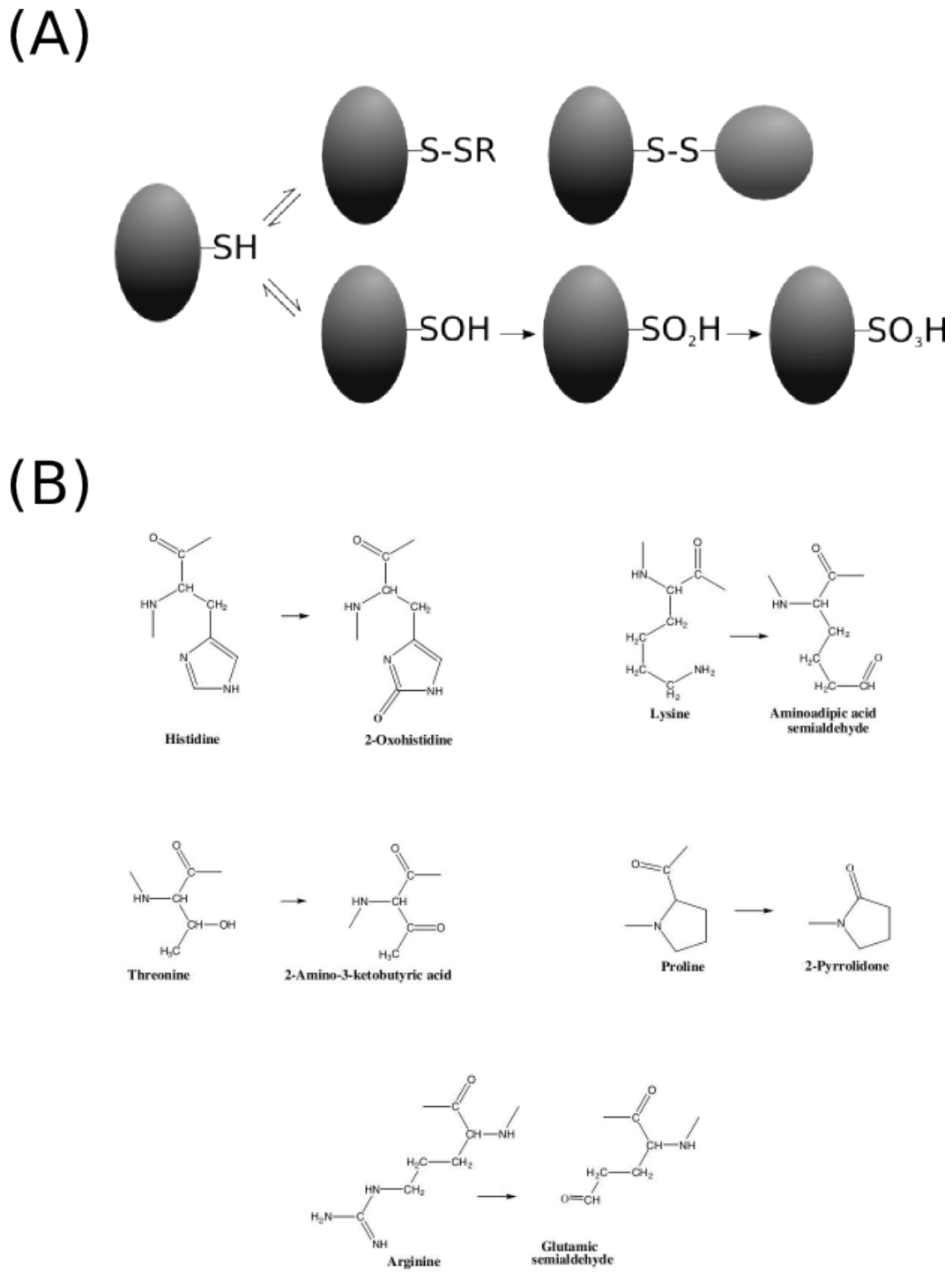

Figure 1.4: (A) Oxidation states of protein cysteine, which can form intramolecular or mixed-disulfide bridges with small molecules or other proteins and can also be reversibly oxidised to sulfenic acid or irreversibly to sulfinic and then sulfonic acid. (B) Many amino acids are oxidised to carbonyl containing molecules, either as free molecules or within proteins.

or on protein carbonylation in mussels (McDonagh et al., 2005), clams (Dowling et al., 2006), yeast (Braconi et al., 2009) or amoeba (Marsano et al., 2010).

There is no study published yet that applies the toolbox of redox proteomics to daphnids. This is not surprising when considering the small amount of molecular studies involving this taxa up to recent years. This gap deserves being filled as the presence of daphnid species in ponds means that OS is already an important part of their ecology, something their exposure to toxicants is likely to increase. Indeed, a relatively high proportion of the molecular literature in daphnids focuses on OS. Such an interest stems from their exposure to high levels of UV, as they can be found in shallow ponds (Borgeraas and Hessen, 2000, 2002), their use as a model species in aquatic toxicology (Barata et al., 2005a,b) as well as their capacity to adapt to oxygen-poor environments (Becker et al., 2011). The application of redox proteomics to daphnids is thus timely. As a first step in this direction, this thesis focuses on the measurement on protein carbonylation using fluorescent labelling and two-dimensional electrophoresis (2DE).

1.6 Protein Carbonyls

Protein carbonyls, as a group, represent the most abundant oxidative modification of proteins (Stadtman, 2006), but they encompass a high structural diversity. This diversity arises from the many reactions that can introduce a carbonyl function in proteins. Primary carbonyls are formed through the direct oxidation of amino acids by ROS, while secondary carbonyls are introduced by the conjugation of lipid and sugar oxidation products, and their derivatives, to the amino and thiol groups of proteins (Stadtman and Levine, 2000). Between these two main categories, there are more than 20 structurally different carbonyl species that are generated by the oxidation of proteins (Madian and Regnier, 2010b). This structural diversity means that carbonyls integrate well the oxidation of proteins

in cells. This is strengthened by the fact that they are produced by different ROS-linked reactions and carbonylation is generally irreversible (Fedorova et al., 2013). Additionally, carbonylation is not just a symptom of oxidative stress, but also plays a role in signal transduction (Wong et al., 2013) and auto-immune syndromes (Fedorova et al., 2013).

It is thus no surprise that many analytical strategies were developed to measure protein carbonylation levels and detect carbonylation sites (Fig. 1.5). Traditionally, protein carbonyls are quantified by labelling with dinitrophenylhydrazine (DNPH), through the formation of a hydrazone linkage, followed by spectrophotometric quantification (Levine et al., 1990). This method requires a large amount of sample and yields no information on the carbonylated proteins themselves. Based on the same derivatisation, immunoaffinity methods have been developed to quantify carbonyls (ELISA, Buss et al., 1997) or detect carbonylated proteins after electrophoretic separation (Western blot, Sultana et al., 2010). While excellent results have been obtained with these methods, they rely on anti-DNP antibodies. They are known to be unreliable and present high noise levels in Western blot, a high level of expertise is thus required to obtain good, reliable results when using them. To circumvent these issues, hydrazine derivatives linked to fluorescent labels were developed to detect and quantify protein carbonyls in gel-based separations. While not replacing DNPH-based spectrophotometric or ELISA approaches for global quantification of protein carbonyls, the use of fluorescent probes enables simple measurement of carbonylated proteins in gel-based approaches. It is even possible to obtain quantitative results if the proper controls are used (Chaudhuri et al., 2006). Four fluorecent labels are used for carbonyl detection: fluorescein-5-thiosemicarbazide (FTC), coumarin hydrazine, BODIPY FL-hydrazide and cyanide dyes (Fedorova et al., 2013). All these enable gel-based proteomics detection of carbonyls without the requirement of transfer to nitrocellulose membranes and immunodetection. Fluorecent scanning of gels, al-

though requiring expensive equipment, is now widely available and requires little expertise.

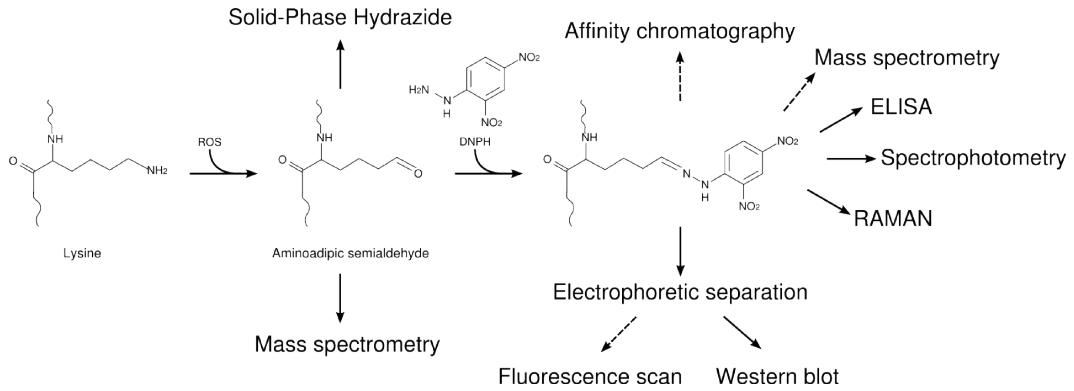

Figure 1.5: Overview of analytical approaches for detection and quantification of protein carbonyls. These can either be direct, or require a specific carbonyl label, DNPH is used as an example in this case. Dashed lines indicate that a label other than DNPH is required for the specified applications.

In parallel to gel-based approaches, many gel-free mass spectrometric methods have been developed in recent years to detect carbonylation sites, either directly (Guo and Prokai, 2011), with labelling (Palmese et al., 2012) or after enrichment (Rauniyar et al., 2010). The high structural diversity of carbonyls means that no single approach can detect equally all carbonyls. For all the methods detailled here, the questions of labelling specificity and detection efficiency are still under investigation (Guo and Prokai, 2011; Bollineni et al., 2013).

In addition to the direct detection of protein carbonyls in a complex matrix, many approaches are available for the isolation of carbonylated proteins, which can then be studied with gel-based or gel-free methods. A hydrazine derivative, biotin-hydrazide being the most common (Madian and Regnier, 2010a), is used to label carbonylated proteins before isolation from the sample by affinity chromatography. Another approach was developed by Roe et al. (2007) using hydrazide-functionalised beads (termed solid-phase hydrazide – SPH) to isolate 4-hydroxynonenal (HNE) derivatised peptides followed by analysis of the modified peptides by mass spectrometry (MS). Chapter 4 expands on their approach and demonstrates its applicability to the isolation of undigested proteins oxidised

by metal-catalysed oxidation (MCO).

Prior to the studies presented herein, no measurement of protein carbonylation in daphnids has been reported in the literature. In line with the central role of oxidative stress in aquatic toxicology (Lushchak, 2011) and concurring interest in protein carbonylation, this thesis focuses on the study of protein carbonylation in *D. magna* using redox-proteomics.

1.7 Methodology

The following two chapters (2 and 3) are interested in the detection and identification of carbonylated proteins in *D. magna*, after exposure to prooxidants copper and paraquat (Chapter 2) and exposure to silver nanoparticles and silver nitrate (Chapter 3). They both follow the same methodological approach, attempting to link the organism-level response to the proteomics measurements.

Whole-organism toxicity of the compounds was tested using the daphnia immobilisation assay, according to OECD protocols (OECD, 2004). This was achieved by exposing neonates ($<$ 24 h) to a wide range of concentrations and visually observe how many of the daphnids were immobilised after 48 h. This binary data was then analysed with a curve-fitting approach (2 parameter log-logistic) to establish the concentration that would cause 50 % immobilisation, the EC50, as shown in Fig. 1.6. The drc package (Ritz and Streibig, 2005) for the statistical software R (R Core Team, 2013) was used for the non-linear modeling and calculation of EC50 values and their confidence intervals.

Following the immobilisation assay, seven days old organisms were exposed to three or four concentrations, from one tenth to one half EC50, of each compound. Seven days old daphnids were chosen over the more usual neonates in order to obtain enough material for the biochemical and proteomics measurements. Indeed, neonates were found to yield about one microgram of protein each after

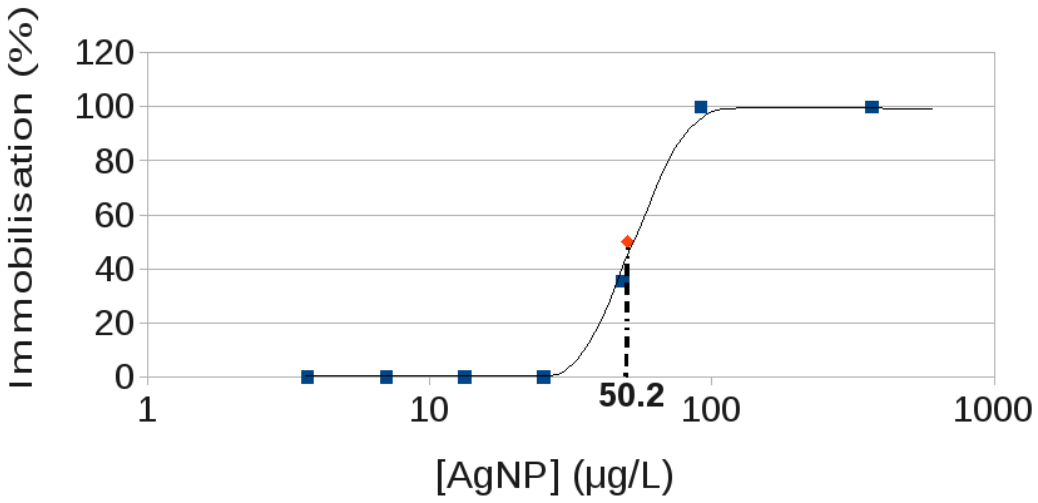

Figure 1.6: Percentage of immobilised neonates as a function of the logarithm of exposure concentration. The presented results are those for 10 months old AgNP (Chapter 3) The observed values are represented by squares and the calculated EC50 by a diamond.

extraction, while this could be increased to 10 µg per organism when using 7 days old daphnids. About one milligram was required per biological replicate to perform the required assays.

The activity of three enzymes was then measured: catalase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glutathione transferase (GST). They were chosen for their involvement in the cellular response to OS. Catalase is one of the main antioxidant enzymes in cells (Matés et al., 1999), it catalyses the reduction of hydrogen peroxide to water and is usually induced when ROS production increases (Lushchak, 2011). GAPDH is a glycolytic enzyme and it is involved in the regulation of many cellular processes (Nicholls et al., 2012). It is known to be sensitive to OS and its expression is increased by oxidative stress. GST is a phase II detoxification enzyme involved, among many other reactions, in the detoxification of lipid peroxidation products and in the reduction of hydroperoxides (Sheehan et al., 2001).

In addition to the measurement of enzymatic activities, two redox-linked PTM were measured: protein thiols and carbonyls. They were respectively labelled with 5-iodoacetamidofluorescein (IAF) and FTC, followed by SDS-PAGE separa-

tion. The expected impact of OS on those two markers are a decrease of protein thiols as they become oxidised and an increase in protein carbonylation (Fig. 1.4).

Proteins from exposed and control *D. magna* were also labelled with FTC prior to 2DE. Protein features were compared between gels using the Progenesis Samespots software, and features of interest (ANOVA p value < 0.05 and fold change $> \pm 1.5$) were picked and sent for MS analysis to identify the proteins they contained. The MS analysis was performed at the Instituto de Technologica Quimica e Biologica (Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal). Bioinformatics were then used to infer the cellular role of the detected proteins in link with the compounds tested.

The third experimental chapter of this thesis (Chapter 4) is methodological. It presents the first application of SPH to the enrichment of whole proteins carbonylated by MCO. Previous applications of SPH focused on the detection of carbonyls introduced by HNE adducts, a secundary carbonyl, on peptides (Rauniyar et al., 2009, 2010; Rauniyar and Prokai, 2011; Roe et al., 2007, 2010). The protocol presented here was developed based on click chemistry literature (von Delius et al., 2010) which often relies on the pH dependent reversibility of hydrazone links formed between a carbonyl and a hydrazine. This then led to reviewing the work of Roe et al. (2007) and expanding its application to undigested proteins. Initial attempts with complex samples did not lead to good results as the long chemical oxidation step led to high levels of proteolysis. Chemically oxidised bovine serum albumin (BSA) was thus chosen as a model protein to demonstrate the principle of the method.

Chapter 2

Application of the redox-proteomics toolbox to *Daphnia magna*

2.1 Introduction

Systems biology, and the omics approaches it is based upon, is gaining in importance in the environmental sciences as its field of application widens away from human and traditional model species. Its presence is thus felt in ecology and ecotoxicology, where there is hope that the gap between subcellular responses and ecosystemic responses to stressors can be linked (van Straalen, 2003). In order to achieve this, environmentally relevant model species need to be used.

In this context, attention is turning towards daphnids, as their ecology is very well studied and omics tools are now applicable to this model organism as the genome of *Daphnia pulex* was recently sequenced (Colbourne et al., 2011) and there is an ongoing project to sequence *D. magna*. The applicability of omics to daphnids is illustrated by the publication in recent years of studies on transcriptomics (Poynton et al., 2012; Jansen et al., 2013; Zeis et al., 2013), proteomics

(Fröhlich et al., 2009; Le et al., 2013; Schwerin et al., 2009; Zeis et al., 2009, 2013) and metabolomics (Taylor et al., 2009; Nagato et al., 2013). This opens the door to the use of hypothesis-free approaches when studying the toxicity of various aquatic contaminants, leading to better insight into mechanisms of toxicity, resistance and interaction between contaminants, as well as potential discovery of novel biomarkers. Daphnids also offer the opportunity to link molecular responses to higher order measurments as their short generation time and well studied reproduction enables multigenerational studies (Shaw et al., 2008).

As the effectors of cells and target of most toxic compounds, proteins present a particular interest in ecotoxicoloy. Their activity has long been studied to understand the impact of toxic compounds or determine the presence of a stress. With the use of proteomics, it is possible to study whole sections of the proteome in one experiment. This does raise the challenge of choosing the proteome to study since no single method can measure all proteins in an organism or tissue (Lemos et al., 2010). In the case of ecotoxicology, redox proteomics, the detection and analysis of redox-based changes in the proteome, is a subproteome of interest as many toxicants are known to alter the redox balance of cells (Sheehan et al., 2010). This is in addition to the growing evidence of the involvement of redox-based post-translational modifications (PTM) in cellular signalling, indicating that looking at changes in the redox-proteome is not only useful in cases of oxidative stress (OS) but also to follow cellular responses to conditions that do not necessarily generate OS (*i.e.* even in sub-stress scenarios). The most widespread of the redox-based modifications, carbonyl formation, occurs as various amino acids are directly oxidised or react with lipid and sugar oxidation products, forming carbonyl containing adducts (Stadtman, 2006). Measurement of protein carbonyl levels is often used as a general marker of oxidative stress in organisms (Almroth et al., 2008; Ching et al., 2009). Although often thought of as a non-specific sign of protein damage, protein carbonylation is known to be selective to certain sites

(Temple et al., 2006) and to be involved in signal transduction (Wong et al., 2010)

In order to show the applicability of redox-proteomics to study the *D. magna* proteome, daphnids were exposed to the prooxidants copper and paraquat, two redox-cycling compounds known to induce oxidative stress (Barata et al., 2005b). Both are also environmentally relevant: copper is a contaminant often released by mining activities (Nagato et al., 2013) and paraquat is a widely used herbicide worldwide.

The approach chosen here was to expose the daphnids to three concentrations of copper and paraquat (1/10 to 1/2 EC50) followed by measurement of enzymatic activities, protein carbonyl and thiol levels and two-dimensional electrophoresis (2DE) of samples labelled with fluorescein-5-thiosemicarbazide (FTC), a carbonyl specific flourescent label (Chaudhuri et al., 2006). Features of interest (showing a significant effect of treatments and a fold change > 1.5) were analysed by MS/MS to identifiy the proteins affected by the treatments in order to gain insight into the mechanism of action of the two compounds. The results show a clear difference between the two prooxidants, although, surprisingly, no sign of OS could be detected.

2.2 Material & Methods

2.2.1 Chemicals

All chemicals were sourced from Sigma-Aldrich Ireland Ltd. (Arklow, Co. Wicklow). Reagents and consumables for proteomic work were sourced from GE Healthcare, UK (Immobiline Drystrips and IPG buffer); Thermo Fisher, Rockford, Il, USA (Unstained protein molecular weight markers); and Bio-Rad, CA, USA (Protein Assay Dye Reagent concentrate).

2.2.2 *Daphnia magna* culture

A clonal culture of *D. magna* was maintained in 4 L plastic aquaria in Elendt M4 medium (Elendt and Bias, 1990) at 20 ± 1 ℃ with a 16:8 light-dark cycle and constant bubbling. Daphnids were fed live *Chlorella vulgaris* (50 000 cells per mL of medium) at least four times a week. Brood stocks were maintained at 50 females per aquarium. Half the medium was renewed two or three times a week. Neonates were removed three times per week. In order to obtain enough tissue for bochemical assays, neonates where maintained for seven days (in 10 mL of medium per neonate), with daily feed, until exposure to the toxicants on day eight.

2.2.3 Immobilisation assay

Immobilisation assays were performed according to OECD (2004) recommendations. Thirty neonates were exposed, in three groups of 10, in glass beakers (40 mL medium) at 20 ± 1 ℃ under a 16:8 light-dark cycle. Immobilisation was observed after 24 and 48 hours. EC50 values were calculated by fitting the results with a two-parameter log-logistic model using the dcr package in R (R Core Team, 2013; Ritz and Streibig, 2005).

2.2.4 Exposures

All exposures were performed for 24 h using 7 days old *D. magna* in 1 L fresh medium in glass beakers at 100 daphnids per beaker in the culture conditions. Daphnids were not fed during the exposures. Daphnids were exposed to three concentrations of $CuCl_2$ (45, 135, 270 µg Cu/L) and three concentrations of paraquat (2, 7 and 13 mg/L). All exposures were repeated four times along with controls. After the exposure, live daphnids were sieved, blotted dry, transferred to a microcentrifuge tube and flash frozen in liquid N_2, they were then stored at

-80 ℃ until homogenisation.

2.2.5 Enzymatic and oxidative lesion assays

As previously (Rainville et al., 2014), 300 µL nitrogen bubbled homogenisation buffer (10 mM tris HCl, pH 7.2, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA and 1 mM PMSF) were added to 100 frozen daphnids in a glass-teflon homogeniser, followed by motor-driven homogenisation for one minute. Homogenates were centrifuged at 14 000 g for 1 h, pellets were discarded and supernatants were aliquoted for subsequent assays. Assays were performed on the day of homogenisation or aliquotes were frozen at -80 ℃ immediately.

Protein concentrations were assessed by Bradford assay (Bradford, 1976) in microtiter plates as per manufacturer's instructions (Bio-Rad Protein Assay Dye Reagent). Enzymatic assays and measurement of protein thiols and carbonyls followed the same protocols as Rainville et al. (2014).

2.2.6 Two-dimensional electrophoresis and protein identification

Protein carbonyls were labelled by adding FTC to freshly prepared tissue homogenates (500 µg) to a final concentration of 1 mM (Chaudhuri et al., 2006). Samples were incubated for 2h in the dark at 4 ℃ before precipitation of proteins with a final concentration of 10 % w/v of trichloroacetic acid (TCA), incubated on ice for 5 min and centrifuged at 11 000 g for 3 min. Pellets were washed twice with 500 µL of ice cold 1:1 ethanol:ethylacetate. Prior to resuspension, they were centrifuged down and dried to make sure no solvent was left in the samples. Rehydration buffer (5 M urea, 2 M thiourea, 2 %w/v CHAPS, 2 % IPG buffer,) was used to solubilise the proteins. Of this, 125 µL, containing 125 µg protein, were loaded unto an Immobiline DryStrip (pH 3-10 NL, 7 cm, GE Healthcare), which

was rehydrated overnight in the dark. Isoelectric focusing was then performed on a PROTEAN IEF system (Bio-Rad), according to the strips manufacturer's recommendation. Hydrated strips were reduced with 2 % w/v dithiothreitol in equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% w/v SDS, 20% v/v glycerol) for 20 min, followed by 2.5 % iodoacetamide in equilibration buffer for 20 min to block thiols. After equilibration, strips were loaded unto 10 % polyacrylamide gels for the SDS-PAGE separation. Gels were scanned using a Typhoon scanner, model 9410 (Amersham Biosciences), with an excitation wavelength of 488 nm and emission of 520 ± 20 nm (bandpass filter). After acquisition of the fluorescence image, gels were stained with colloidal coomassie (Dyballa and Metzger, 2009), and images were acquired with a GS-800 Calibrated Densitometer (BioRad, Hercules, CA, USA). Image analysis was performed with the Progenesis SameSpots software (Nonlinear Dynamics Limited, UK). Experiments were defined by compound, exposure concentrations were the treatments. Spots were considered of interest when showing a 1.5 fold change between treatments as well as being statistically significant ($p < 0.05$ in ANOVA). Interesting, well resolved spots of sufficient intensity were then selected for mass spectrometric analysis. Features from the fluorescence images were considered interesting only if they could be matched to a coomassie stained feature of significant intensity.

Those were manually excised using clean pipette tips and in-gel digested with trypsin according to Almeida et al. (2010). Extracted peptides were loaded onto a R2 micro column (RP-C18 equivalent) where they were desalted, concentrated and eluted directly onto a MALDI plate using α-cyano-4-hydroxycinnamic acid as the matrix solution in 50 % acetonitrile and 5 % formic acid. Mass spectra of the peptides were acquired with positive reflectron MS and MS/MS modes using a MALDI-TOF/TOF MS instrument (4800 plus MALDI TOF/TOF analyzer) with exclusion list of the trypsin autolysis peaks (842.51, 1045.56, 2211.11 and 2225.12). The collected MS and MS/MS spectra were analysed in combined

mode by using the Mascot search engine (version 2.2; Matrix Science, Boston, MA) and the NCBI database restricted to 50 ppm peptide mass tolerance for the parent ions, an error of 0.3 Da for the fragments, one missed cleavage in peptide masses, and carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. No taxonomy restrictions were applied. The identified proteins were only considered if a MASCOT score above 95% confidence was obtained ($p < 0.05$) and at least one peptide was identified with a score above 95% confidence ($p < 0.05$). This analysis was conducted by the Analytical Services Unit, Instituto de Tecnologia Química e Biológica (ITQB), New University of Lisbon, Lisbon, Portugal.

2.2.7 Data analysis

Images from 1DE were analysed with the Quantity One software (Bio-Rad) to obtain a single trace measurement per lane. Fluorescence values were normalised for loading by dividing them with their trace coomassie value. Statistical analysis of enzymatic and PTM assays were performed by one-way ANOVA with a Holm-Sidak post-hoc test (versus control), using the Sigmaplot 10.0 software (Systat Software, Inc.).

2.2.8 Hypothetical proteins

Hypothetical proteins (HP) identified by MS/MS were studied using bionformatics tools to find their potential function. Blastp (Altschul et al., 1997) and DELTA-BLAST (Boratyn et al., 2012) from the National Center for Biotechnology Information (`http://blast.ncbi.nlm.nih.gov/Blast.cgi`) were used to identify sequence similarity with known proteins. In addition, conserved sites, domains and families present in the HP were studied with Interproscan (Quevillon et al., 2005) from the European Bioinformatics Institute (`http://www.ebi.ac.uk/Tools/pfa/iprscan/`). Results shown below include the highest scoring,

non-hypothetic protein from blastp, as well as the main (non-redundant) domains and families identified from Interproscan. In the present study, those were always in accordance with results from DELTA-BLAST, which are not presented.

2.3 Results

2.3.1 Classical endpoints

In order to choose biologically meaningful concentrations for the biomarker and proteomic measurements, immobilisation assays were performed using neonates. The calculated EC50 were much higher for paraquat (22.58 mg/L; 95 % CI: 20.27–25.15 mg/L) than for copper chloride (415 µg Cu/L; 95 % CI: 317–544 µg Cu/L).

From those results, sublethal concentrations were chosen to expose the daphnids in order to obtain meaningful results from the biochemical measurements. The activity of the enzymes catalase, glutathione transferase (GST) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured after exposures and effects were observed only in a decrease of catalse activity in daphnids exposed to 2 and 7 mg/L paraquat (Fig. 2.1). Levels of protein thiols and carbonyls were also not affected significantly by the treatments (data not shown)

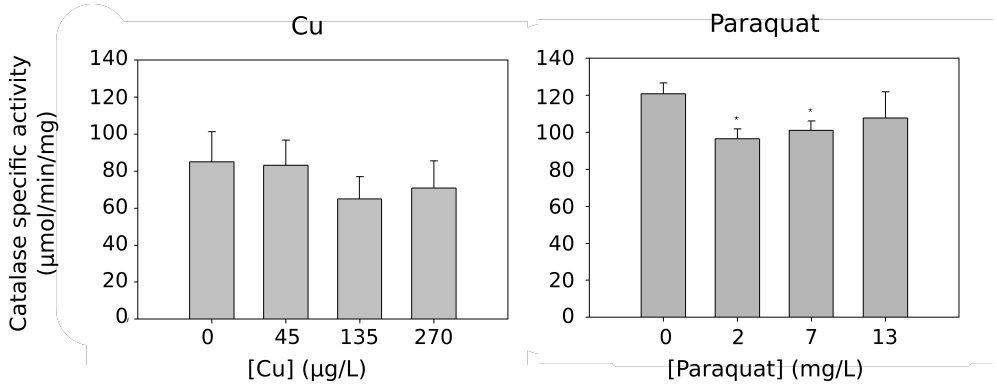

Figure 2.1: Catalase activity of seven days old daphnids after exposure to copper and paraquat. (One-way ANOVA: *: $p < 0.05$; **: $p < 0.01$; ***:$p < 0.001$)

2.3.2 2DE

A total of 30 features were significantly modified by copper exposures and showed a fold change of at least 1.5. With increasing copper concentration, nine showed an increase in volume at the protein level, 11 a decrease and one a varying pattern with concentration. Additionnaly, six showed a decrease in carbonylation with increasing Cu, while 3 showed a decrease in carbonylation concomitant with a decreased volume of the protein stain for the same feature. Two features presented an increase of carbonylation (FTC labelling volume) alone and two an increased carbonylation concomitant with increased volume of protein stain.

In paraquat exposed samples, only two features showed significant changes of at least 1.5 fold. One was an increase in protein stain volume with concentration (f26) while the other showed a decrease in carbonyl content with concentration (f24).

All features of interest (shown in Fig. 2.2) were picked for protein identification by MSMS

2.3.3 Protein identification

Of the 32 features of interest found with 2DE, 19 could be successfully identified by MS/MS analysis (see Table 2.1). Among those, six were identified as proteins of known function. Of these, actin and an actin related protein were identified, along with proteins related to protein synthesis (translational elongation factor 2, TEF-2) and degradation (trypsin), reproduction (vitellogenin fused with SOD) and glucose metabolism (2-phospho-D-glycerate hydrolase).

In addition to these, 13 proteins were identified as hypothetical or uncharacterised, eleven of which were from the recently completed *D. pulex* genome (Colbourne et al., 2011). Among these, f3 and f4 contained the same protein, probably because that they are part of a feature train, leaving twelve sequences of unknown function. They were analysed with pblast, DELTA-BLAST and In-

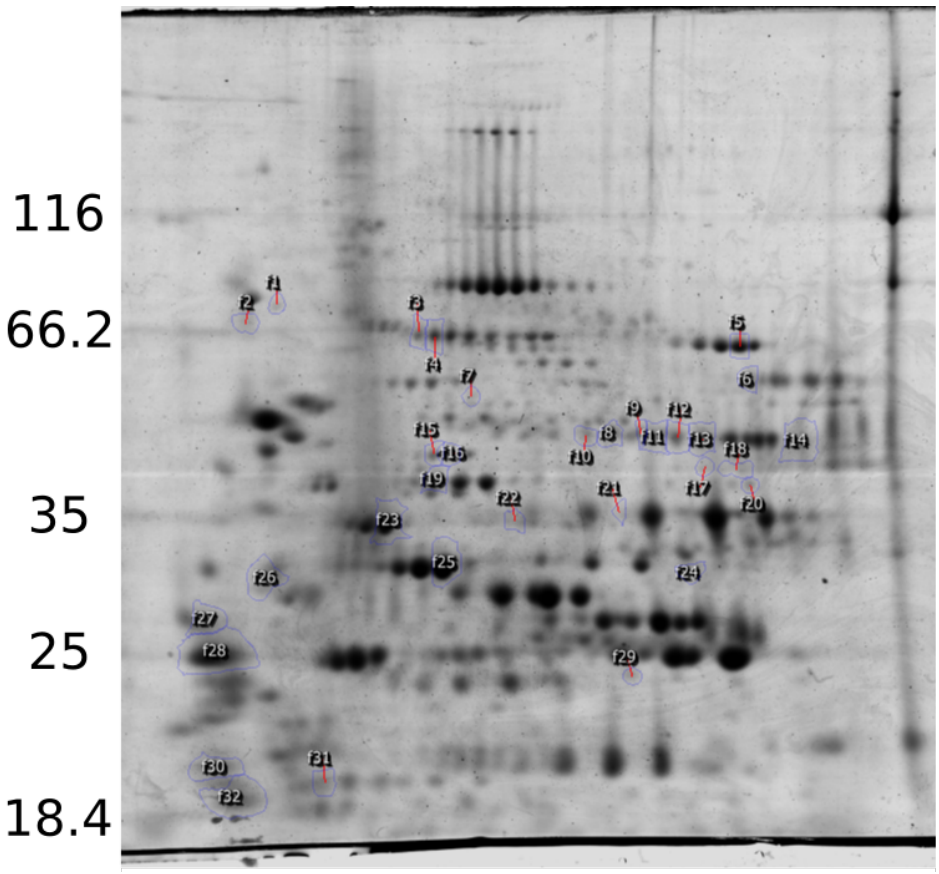

Figure 2.2: 2DE separation of 100 µg of *Daphnia magna* proteins, colloidal coomassie stained. Features modified by $CuCl_2$ and paraquat exposures are shown ($p < 0.05$ and > 1.5 fold change)

terproscan to identify a putative function for the proteins. The results from this analysis are shown in Table 2.2. The possible biological functions of those proteins include proteolysis (neural endopeptidase 24.11), reproduction (vitellogenin fused with SOD), stress response (members of the HSP70 family), actin filament assembly (alpha actinin), glycolysis (fructose 1,6-biphosphate aldolase), energy metabolism (arginine kinase) and protein folding (peptidyl-prolyl cis-trans isomerase).

Seventeen of the identified features were affected by copper exposures, while only two were affected by paraquat exposures. Fold changes of the identified features following treatments are shown in Table 2.3.

Table 2.1: Proteins affected by prooxidant exposure and identified by LC-MS/MS

Feature	**Protein**	**Organism**	**Accession number**	**MW** (kDa) (Measured / Predicted)	**Protein Score**[a] **/ Protein C.I.** (%)	**Total Ion Score / Total Ion C.I.** (%)	**Sequence coverage** (%)	**Number of peptides**[b]
f2	hypothetical protein DAPPUDRAFT_200882	*Daphnia pulex*	gi|321461668	73/75	362/100	357/100	7	2
f3	hypothetical protein DAPPUDRAFT_213992	*Daphnia pulex*	gi|321465380	69/228	153/100	153/100	10	2
f4	hypothetical protein DAPPUDRAFT_213992	*Daphnia pulex*	gi|321465380	69/228	162/100	144/100	11	2
f6	hypothetical protein DAPPUDRAFT_313764	*Daphnia pulex*	gi|321474716	60/170	244/100	244/100	10	1
f7	uncharacterized protein LOC393586	*Danio rerio*	gi|41055387	57/71	320/100	388/100	27	3
f8	hypothetical protein DAPPUDRAFT_301437	*Daphnia pulex*	gi|321457382	52/56	213/100	208/100	11	1
f10	hypothetical protein DAPPUDRAFT_313359	*Daphnia pulex*	gi|321475312	51/73	160/100	144/100	27	2
f15	hypothetical protein	*Amblyomma maculatum*	gi|346468137	48/71	168/100	149/100	5	2
f16	hypothetical protein DAPPUDRAFT_99081	*Daphnia pulex*	gi|321473812	48/105	550/100	448/100	26	3
f21	vitellogenin fused with superoxide dismutase	*Daphnia magna*	gi|95113632	41/225	339/100	327/100	15	2
f22	2-phospho-D-glycerate hydrolase	*Daphnia magna*	gi|41394397	39/40	746/100	700/100	54	6
f23	actin related protein 1	*Acyrthosiphon pisum*	gi|217330650	39/42	659/100	613/100	54	4
f24	hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	gi|321469582	34/39	209/100	209/100	11	1
f25	hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	gi|321478205	34/40	915/100	789/100	63	7
f28	trypsin 208, partial	*Daphnia magna*	gi|403311427	26/26	551/100	526/100	34	3
f29	translational elongation factor-2	*Daphnia magna*	gi|262303391	25/81	550/100	526/100	22	3
f30	hypothetical protein DAPPUDRAFT_231271	*Daphnia pulex*	gi|321463635	19/23	166/100	151/100	30	1
f31	actin	*Timema boharti*	gi|323435224	18/31	502/100	439/100	41	3
f32	hypothetical protein DAPPUDRAFT_230174	*Daphnia pulex*	gi|321478432	17/33	115/100	115/100	4	1

[a] The protein score probability limit (where p <0.05) is 86.

[b] Number of MS/MS patterns assigned to peptides with confidence interval above 95 %.

Table 2.2: Blast and interproscan search of hypothetical proteins modified by copper and paraquat exposures

Feature	**Accession Number**	**Best hit**[a] (protein (*species*), E-Value, Identity %)	**Families and Domains**[b]	**GO functions**[b]
f2	gi|321461668	Neural Endopeptidase 24.11 (*Bombyx mori*) E: 8e-170, Identity: 40%	Peptidase M13	metalloendopeptidase activity; proteolysis
f3,f4	gi|321465380	Vitellogenin fused with superoxide dismutase (*Daphnia magna*) E: 0.0, Identity: 52%	Superoxide dismutase (Cu/Zn)/chaperones; von Willebrand factor, type D Domain; Vitellinogen, open beta-sheet; Vitellinogen, beta-sheet N-terminal	Superoxide metabolic process; Metal ion Binding; Oxidation-reduction Process; Lipid transport activity; Lipid transport
f6	gi|321474716	Vitellogenin-like protein (*Lepeophtheirus salmonis*) E: 3e-106, Identity: 24%	von Willebrand factor, type D Domain; Vitellinogen, superhelical; Vitellinogen, beta-sheet N-terminal	Lipid transport activity; Lipid transport
f7	gi|41055387	HSC71 (*Krypolebias marmotus*) E: 0.0, Identity: 95%	Heat shock protein 70 family	
f8	gi|321457382	Sb:cb283 protein, partial (*Danio rerio*) E: 0.0, Identity: 59%	Leucine aminopeptidase/peptidase B	aminopeptidase activity; cytoplasm; metalloexopeptidase activity; protein metabolic process; manganese ion binding
f10	gi|321475312	heat shock protein 70 (*Moina mongolica*) E: 0.0, Identity: 89%	Chaperone DnaK; Heat shock protein 70 family	ATP binding; protein folding; unfolded protein binding
f15	gi|346468137	heat shock protein 70kDa, partial (*Diguetia signata*) E: 0.0, Identity: 81%	Heat shock protein 70 family	
f16	gi|321473812	GG12661 (*Drosophila erecta*) E: 0.0, Identity: 84%	Alpha-actinin; EF-hand domain	actin filament bundle assembly; actin crosslink formation; calcium ion binding
f24	gi|321469582	fructose 1,6-bisphosphate aldolase (*Bombyx mori*) E: 0.0, Identity: 80%	Fructose-bisphosphate aldolase, class-I	fructose-bisphosphate aldolase activity; glycolysis
f25	gi|321478205	Arginine Kinase (*Artemia franciscana*) E: 0.0, Identity: 79%	ATP:guanido phosphotransferase; Glutamine synthetase/guanido kinase, catalytic domain	catalytic activity; kinase activity
f30	gi|321463635	fk506-binding protein (*Aedes aegypti*) E: 8e-93, Identity: 63%	Peptidyl-prolyl cis-trans isomerase, FKBP-type; EF-hand domain	protein folding; calcium ion binding
f32	gi|321478432	chymotrypsin-like protein (*Daphnia pulex*) E: 3e-75, Identity: 50%	Peptidase S1A, chymotrypsin-type; Trypsin-like cysteine/serine peptidase domain	serine-type endopeptidase activity; proteolysis

[a] Best hit results are obtained using a blastp search (Altschul et al., 1997).
[b] Families, domains and GO functions were obtained using Interproscan (Quevillon et al., 2005).

Table 2.3: Fold change of feature volumes of identified proteins[a]

	Cu (coomassie/fluorescence)			**Paraquat** (coomassie/fluorescence)		
Protein (feature)	45 µg/L	135 µg/L	270 µg/L	2 mg/L	7 mg/L	13 mg/L
Neural Endopeptidase 24.11 (f2)	-1.9/-2.0	-1.6/-1.8	-2.0/-2.5	– / –	– / –	– / –
Vitellogenin fused with superoxide dismutase (f3)	– / 2.2	– / 2.6	– / 2.4	– / –	– / –	– / –
Vitellogenin fused with superoxide dismutase (f4)	– / 2.0	– / 2.3	– / 2.5	– / –	– / –	– / –
Vitellogenin-like protein (f6)	1.5 / 1.0	1.7 / 1.6	2.1 / 1.6	– / –	– / –	– / –
HSC71 (f7)	-1.2/ –	-2.1/ –	-2.1/ –	– / –	– / –	– / –
Sb:cb283 protein, partial (f8)	-1.3/-1.1	-1.4/-1.3	-1.8/-1.6	– / –	– / –	– / –
Heat shock protein 70 (f10)	– /-1.3	– /-1.4	– /-1.7	– / –	– / –	– / –
Heat shock protein 70kDa, partial (f15)	-1.4/ –	-2.2/ –	-3.0/ –	– / –	– / –	– / –
GG12661 (f16)	-1.3/ –	-1.8/ –	-2.6/ –	– / –	– / –	– / –
Vitellogenin fused with superoxide dismutase (f21)	1.2 / –	1.6 / –	1.5 / –	– / –	– / –	– / –
2-phospho-D-glycerate hydrolase (f22)	– / –	– / –	– / –	– /-1.6	– /-1.4	– /-1.7
Actin related protein 1 (f23)	-1.2/ –	1.0 / –	1.4 / –	– / –	– / –	– / –
Fructose 1,6-bisphosphate aldolase (f24)	– / –	– / –	– / –	-1.2/ –	1.1 / –	1.4 / –
Arginine Kinase (f25)	– /-1.4	– /-1.7	– /-1.6	– / –	– / –	– / –
Trypsin 208, partial (f28)	-1.2/-1.2	-1.7/-1.6	-1.8/-1.9	– / –	– / –	– / –
Translational elongation factor-2 (f29)	-1.2/ –	1.3 / –	1.5 / –	– / –	– / –	– / –
fk506-binding protein (f30)	– /-1.5	– /-1.4	– /-2.0	– / –	– / –	– / –
Actin (f31)	-1.1/ –	-1.4/ –	-1.9/ –	– / –	– / –	– / –
Chymotrypsin-like protein (f32)	– /-1.3	– /-1.4	– /-2.0	– / –	– / –	– / –

[a] Fold changes are relative to control and given only for treatment where the feature was significantly changed (ANOVA p <0.05).

2.4 Discussion

2.4.1 Whole organism Toxicity

The measured EC50 for Cu is relatively high compared to previously published toxicity values for *D. magna* (Barata et al., 2005b). This is due to the presence of EDTA in the M4 medium, a well-known phenomenon that can hamper toxicity evaluation of metal containing samples (Guilhermino et al., 1997). As this study is focused in determining the usefulness of redox-proteomics in studying the mechanism of toxicity of Cu, it was felt that choosing a copper concentration

relatively to its biological effect, the observed EC50 in M4 medium, should not bias results compared to a chelating agent-free medium in which a similar criterion would be used. In the case of paraquat, the measured EC50 conforms well with previously published data (USEPA, 2013).

2.4.2 Biomarkers

Following exposure to three concentrations of Cu and paraquat, the enzymatic activity of GST, catalase and GAPDH were measured along with the relative content of protein carbonyls and thiols. Of these, only catalase activity was affected by the treatments. Catalase was reduced following paraquat exposures (Fig. 2.1). It is surprising to find so few effects at the enzymatic level as previous studies have shown changes in enzymatic activities, although with younger daphnids and a longer exposure time (Barata et al., 2005b). Catalase and GST have also been shown to increase in activity following exposure of *D. magna* to environmental samples rich in Zn and Cu (Yoo et al., 2013).

As neither the measured enzymatic activities nor oxidative lesions of proteins were strongly affected by the treatments, we infer that no OS occured during the treatments. Although surprising from the relatively high exposure concentrations used and known mechanism of actions of both compounds, this is confirmed by 2DE analysis. At the feature level, a significant reduction in FTC labeling is more frequent than an increase. Such a reduction indicates that cells managed to compensate for the likely higher concentrations of ROS resulting from the exposure to prooxidants. As this lack of OS is common to both toxicants, it is unlikely to be a result of the chelation of Cu ions by EDTA. A possible explanation is the use of light bubbling during exposure of the daphnids. As this is not often reported in the literature, it is not possible to infer its impact on oxidative stress following toxicant exposure, but as it affects O_2 levels, it may impact on cell respiration, OS generation and anti-oxidant defenses.

2.4.3 Two-dimensional electrophoresis

A total of 30 features were significantly affected ($p < 0.05$ and 1.5 fold change) by the copper exposures, while only two were affected by paraquat. Most of these presented a change only of the protein or carbonyl content, indicating that those two parameters changed mostly independently. Five features did present parallel changes of protein and carbonylation levels, meaning that the protein level was modified while the carbonylation level of individual proteins was not markedly altered. Nine of the 32 features were detected as modified at the protein carbonyl level only, increasing by almost a third the number of proteins of interest detected, without requiring more sample or extra experiments. There is thus a strong gain in labelling redox-linked PTM in ecotoxicological studies. This is particularly interesting as no OS was detected in this study, and seven of these nine features showed a reduction in carbonyl content. The information gained can thus be more general than an identification of the targets of OS. This is not surprising as changes in protein carbonyl levels are to be expected in contexts other than OS. Indeed, carbonylation is specific to certain sites on proteins (Temple et al., 2006) and there is a growing indication that carbonylation is involved in signal transduction (Wong et al., 2010).

FTC labelling also enabled us to confirm five of the detected features for which the carbonyl and protein content were significantly altered and fold changes presented similar values. This cannot be expected for all features that change only at the protein level, as many proteins may not be carbonylated (or only weakly) and thus not detected by FTC. The opposite does not hold in this study, as features that showed changes at the FTC level but could not be seen with the colloidal coomassie stain were not considered here, as it was not possible to pick the feature for MS/MS.

2.4.4 Protein Functions

Nineteen proteins could be identified by MS/MS from the features of interest. Of those, 17 were affected by copper, twelve of which were identified as hypothetical or uncharacterised proteins. Those were further studied with blastp, DELTA-BLAST and InterproScan to infer their possible biological functions. For all proteins, the three search engines yielded similar functions, which are detailed in Table 2.2. The following discussion uses the MS/MS identifications and inferred biological functions of hypothetical proteins to gain insight into the impact of copper exposure in *D. magna*.

The two most represented functional grounps among the identified proteins are vitellogenins (features f3, f4, f5 and f21) and proteolytic enzymes (f2, f8, f28, f32). Vitellogenins (vtg) are involved in egg development in oviparous species, including the development of parthenogenetic eggs in daphnids (Kato et al., 2004). Features 3 and 4 appear to be composed of DmaVTG2 (Tokishita et al., 2006) and feature 21 was identified as DmaVTG1 (Kato et al., 2004). These two vitellogenins contain a superoxide dismutase (SOD) domain, though of weak activity (Kato et al., 2004). Feature 6 is similar to a vitellogenin from the salmon louse (Table 2.2). Taken together, these four features show an increase at the protein (f6 and f21) and/or carbonyl level (f3,f4 and f6). The observed results may be linked to changes in vtg maturation or increased expression (Hannas et al., 2011). Vitellogenins are extensively modified prior to their uptake by developing eggs and this maturation process is known to be affected by stressors (Gündel et al., 2007). The mechanistic significance of changes in vtg expression or maturation is not known in crustaceans, although it is of interest that the changes in protein content and carbonylation levels observed in this study vary markedly from those of a previous study on silver nanoparticles and silver nitrate conducted in our laboratory (Rainville et al., 2014). This is in agreement with previous publications indicating that the vitellogenin proteome is an easily accessible and

specific marker of physiological changes in vertebrates and invertebrates alike (Gündel et al., 2007; Jubeaux et al., 2012), enabling the identification of protein expression signatures (PES) with a potential to differentiate stressors.

Another four features were shown to contain proteolytic enzymes (f2, f8, f28, f32). Two of these are digestive enzymes (trypsin, f28, and chymotrypsin, f32) normally excreted in the daphnid gut and involved in protein digestion. The function of the other two enzymes is harder to clarify as they are members of the peptidase M13 family (f2) and the Leucine aminopeptidase family (f8). Members of the M13 family are metalloendopeptidases that cleave small substrates of up to 40 amino acids, their functions vary from protein digestion in bacteria to cleavage of small signaling peptides in mammals (Rawlings et al., 2012). Leucine aminopeptidases are cytoplasmic enzymes involved in the degratadion of intracellular peptides (Rawlings et al., 2012). The feature volume of all four proteases was reduced with increasing copper concentration, for features 2, 8 and 28, the reduction is observed at the protein and carbonyl level while for f32 (chymotrypsin-like protein) the reduction is only at the carbonyl level.

As a group, proteolytic enzymes have often been detected in omics studies on daphnids. Using proteomics, Schwerin et al. (2009) found that digestive proteases were repressed in cold-acclimated *D. pulex*. In a transcriptomics study on *D. magna*, Poynton et al. (2007) found that various proteases were downregulated by Cd exposure while others were upregulated by Cu or Zn. Interestingly, they found an upregulation of a trypsin precursor mRNA and an aminopeptidase mRNA following copper exposure, while we observe a reduction in protein levels for a trypsin and a leucine aminopeptidase under exposure to the same metal. De Coen et al. (1998) observed a reduction of trypsin activity in *D. magna* exposed to various metals and organic compounds, in close correlation with the measured EC50 for immobilisation. Digestive enzyme activity is thus known to respond to toxic stress, although not necessarily by a decrease (De Coen and Janssen,

1997). Although this also held true in their study for esterase and β-galactosidase activities, only trypsin was detected as having a reduced protein level in our study.

Three members of the heat shock protein 70 (HSP70) family were found with the sequence similarity search (f7, f10, f15). HSP70 are chaperones involved in protein folding and are either constitutively expressed or induced by stress in order to maintain protein integrity (Mathew and Morimoto, 1998). In *D. magna* HSP70 levels have been shown to increase under exposure to diclofenac (Haap et al., 2008) and cadmium (Haap and Köhler, 2009). In the present study, two of the identified HSP70s (f7 and f15) showed lower protein levels and the third (f10) presented lower carbonylation levels. All three were found to have a reduced mass of about 50 kDa (Table 2.1), which may be a result of proteolytic degradation of the proteins during sample preparation.

Interestingly, in addition to the three identified HSP70, another protein involved in protein folding was detected as having a reduced carbonyl content (f30). This protein has a similar sequence to a peptidyl-prolyl cis-trans isomerase (cyclophilin). Cyclophilins are known to be involved in the heat shock response along with HSP and proteases (Mathew and Morimoto, 1998). The observed general reduction of those three functional catregories of proteins points to a general reduction of the heat-shock response in daphnias as a result of copper exposure. Although surprising at first glance, many studies show that lower basal levels of HSP and lower induction following stress are linked to better resistance to the toxicity of metals (Haap and Köhler, 2009). A more in depth study would be required to elucidate the meaning of the observed results, but it points to the interesting behaviour of the heat-shock response in daphnids, which is not as systematically associated to stress as could be expected (Pauwels et al., 2010).

Another three proteins have potential biological functions related to the cytoskeleton, all showing changes at the protein level with increasing Cu concentrations. The protein content of features 16 and 31 decreased while it increased

for f23. Features 23 and 31 contain actin-related proteins and f16 contains a member of the alpha-actinin family. While actins are the basic unit of microfilaments (actin filaments), alpha-actinin is involved in cross-linking actin filaments together or with proteins associated to the cell membrane (Djinović-Carugo et al., 1999). Actin filaments are one of the main component of the cytoskeleton and the muscle fiber. They are involved in intracellular trafficking and cellular motility. Schwerin et al. (2009) have found that many forms of actin are upregulated in *D. pulex* acclimated to 10 ℃. Actin expression has been shown to decrease in *D. magna* acclimated to the presence of predators at the protein (Pijanowska and Kloc, 2004) and mRNA level (Schwarzenberger et al., 2009). Actin thus reacts to different stressors in daphnids, but does present some level of specificity to the stressor as its mRNA levels were shown not to vary under ibuprofen exposure (Heckman et al., 2006). Interestingly, the two actins observed to be modified in this study show opposite expression patterns. The isoforms of actin would need to be identified to better understand the significance of these changes.

The last two proteins that changed after copper exposure are arginine kinase (f25) and TEF2 (f29). Arginine kinase is the enzyme responsible for the only phosphagen system found in arthropods (Ellington, 2001). Arginine kinase enables phosphate, proton and ATP buffering in cells by reversibly transferring energy-rich phosphate bonds between arginine and ATP, thus contributing to intracellular energy transport (Ellington, 2001). The observed reduction in carbonylation of arginine kinase would indicate that the protein is either cycled at a higher rate, thus not accumulating as many carbonyls, or somehow protected from carbonylation. In both cases, this would maintain a higher level of activity. In another study, arginine kinase mRNA was found to be downregulated in *D. magna* exposed to ZnO nanoparticles (Poynton et al., 2011).

Finally, TEF2, a protein involved in protein synthesis at the ribosomal level, presented an increased coomassie stain level after copper exposure. This is an

opposite response to that observed by Connon et al. (2008) in *D. magna* exposed to Cd. They found an increase of TEF2 mRNA at low concentrations along with a decrease at high [Cd]. This was concomitant with changes of many ribosomal proteins mRNA, alhough the direction of change was dependent on the specific mRNA. Changes in protein systhesis are expected in stressed organisms although, the impact of a reduction of TEF2 levels in *D. magna* are not known.

Overall, there are few indications of a specific response to Cu within the present results. The same functional categories of proteins or genes identified here have often been found to react to stressors in daphnids (Poynton et al., 2007; Connon et al., 2008; Poynton et al., 2008a) This points to a relatively general stress reponse rather than a specific response to copper. As highlighted by Poynton et al. (2008a), the reduction in proteases may indicate a specific toxicity to the hepatopancreas but, as we observe an increase in vtg fragments, also synthetised in the hepatopancreas, a more focused experimental approach would be required to determine the mode of action of copper in *D. magna*.

Although disapointing, this result is balanced by the fact that a very different response was obtained for paraquat in the present experiment. Indeed, no feature was common to the two toxicants, and as a slight mortality was observed at the highest concentration for both compounds (results not shown) there was a high level of physiological stress. Yet, only two features were shown to be modified by the paraquat exposure, both were identified by MS/MS, one (f22) as 2-phospho-D-glycerate hydrolase (enolase), the other (f24) as a hypothetical protein from the *D. pulex* genome, with a high level of identity with fructose 1,6-bisphosphate aldolase (aldolase). Both enzymes are part of the glycolytic pathway. Enolase saw its carbonylation levels decrease significantly upon paraquat exposure, while aldolase protein levels increased with concentration. In both cases, this would lead to higher activity of the protein, thus increased glycolysis, indicating higher energetic demands for the daphnids. Paraquat acts as an electron acceptor in the

mitochondrion, subsquently leading to superoxide formation and OS (Vicente et al., 2001). This leads to an increased leaking of protons within the mitochondria, resulting from lipid peroxidation of the mitochondrial membrane (Vicente et al., 2001). This is in contradiction with our results indicating no significant amount of OS to the *D. magna* proteins. Although the slightly decreased catalase activity observed may be linked to increase ROS production (Mayo et al., 2003), it appears more likely that the cell is trying to circumvent a loss of mitochondrial efficiency by stimulating glycolysis, rather than being under strong oxidative stress.

2.5 Conclusion

This study was concerned with the impact of copper and paraquat on *D. magna* at the protein level. Classical biomarkers and measurement of oxidative lesions of proteins did not respond significantly to the two prooxidants. Despite the lack of observed OS, 2DE of samples labelled for protein carbonyls showed markedly different responses to the two compounds, with no feature being modified by both. Subsequent mass spectrometry analysis led to the identification of 17 proteins whose abundance and/or carbonylation were affected by copper and of two proteins affected by paraquat. In copper exposed samples, a reduction of the heat-shock response was observed (less abundant proteases, HSP70 and cyclophilin) along with changes in vitellogenins, actin filament-linked proteins, protein metabolism and the phosphagen system. Following paraquat exposures, proteins involved in glycolysis were found to be more abundant or less carbonylated, indicating a likely increase of glycolysis. Taken together this indicates that the organisms managed to mitigate the typical prooxidant effect of the toxicants, but not to the point of avoiding major stress, as mortality was observed at the highest concentration of both compounds. This study shows the usefulness of

labelling redox-related lesions in proteins even in the absence of measurable oxidative stress, as nine of the features discovered by 2DE showed changes at the carbonyl level and not the protein abundance. Labelling with FTC also led to the confirmation of five of the features for which changes were observed at the protein level, reinforcing their statistical significance.

Chapter 3

Proteomic evaluation of citrate-coated silver nanoparticles toxicity in *Daphnia magna*

The following chapter was submitted as an article to Analyst on November 9th, 2013. It was sent for peer review on November 21st, 2013 and published on the 7th of March 2014. The article is included here, with no modifications, along with the supporting informations.

Analyst

PAPER

Proteomic evaluation of citrate-coated silver nanoparticles toxicity in *Daphnia magna*†

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Recent decades have seen a strong increase in the promise and uses of nanotechnology. This is correlated with their growing release in the environment and there is concern that nanomaterials may endanger ecosystems. Silver nanoparticles (AgNPs) have some of the most varied applications, making their release into the environment unavoidable. In order to assess their potential toxicity in aquatic environments, the acute toxicity of citrate-coated AgNPs to *Daphnia magna* was measured and compared to that of $AgNO_3$. AgNPs were found to be ten times less toxic by mass than silver ions, and most of this toxicity was removed by ultracentrifuging. At the protein level, the two forms of silver had different impacts. Both increased protein thiol content, while only AgNP increased carbonyl levels. In 2DE of samples labelled for carbonyls, no feature was significantly affected by both compounds, indicating different modes of toxicity. Identified proteins showed functional overlap between the two compounds: vitellogenins (vtg) were present in most features identified, indicating their role as a general stress sensor. In addition to vtg, hemoglobin levels were increased by the AgNP exposure while 14-3-3 protein (a regulatory protein) carbonylation levels were reduced by $AgNO_3$. Overall, this study confirms the previously observed lower acute toxicity of AgNPs, while demonstrating that the toxicity of both forms of silver follow somewhat different biologic pathways, potentially leading to different interactions with natural compounds or pollutants in the aquatic environment.

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1 Introduction

The last decade has seen a sharp increase in the number of studies concerned with the environmental impact of engineered nanoparticles (NP). This concern is fueled by the increasing variety of uses for NP, and their continued development. This is leading to increased NP usage and release in the environment.[1]

The novel properties of materials at the nanoscale makes evaluation of their toxicity particularly challenging. Size, surface chemistry, coating, method of suspension and composition all affect the laboratory-based measurement of their toxicity. Although generation of reactive oxygen species (ROS) is a frequent component of NP toxicity,[2] the impressive range of NP being manufactured currently means that no general rule can be used to infer their toxicity.

Silver NP (AgNP) are the most widely used commercial nanoparticles, with applications in textiles, medical equipment and household products, mostly involving their antibacterial properties.[3] Their widespread use leads to their release into the environment through their leakage and disposal.[4] Additionally, their behaviour in the environment and in wastewater treatment plants is still a subject of study.[4,5] There is thus a need to understand their interaction with biota, especially in the aquatic environment, where the particles will most likely end up.

As many studies highlight their potential toxicity,[6–8] efforts to discern their mechanism of action are becoming more frequent. Yet, the prior knowledge required to properly deploy traditional, hypothesis-based, biomarkers to study AgNP toxicity is still lacking. There is thus an interest in exploiting omics-based tools to gain a better understanding of their toxicity mechanism and to discriminate it from that of their bulk components. This strategy was used by Poynton *et al.*[8] as they applied transcriptomics to identify changes in gene expression following exposure to citrate-coated AgNP, PVP-coated AgNP as well as silver nitrate. This led them to conclude that toxicity of AgNP differed from that of silver nitrate at the mechanistic level and, to a lesser extent, varied with different NP coatings.

In addition to knowledge gained from gene expression studies, the study of proteins is of particular interest in toxicology as they are the principal quantitative targets of toxicity and their covalent modification can lead to various changes in metabolism and cell signaling.[9] Additionally, proteins are the

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first targets of the reactive oxygen species (ROS) that accumulate in cells experiencing oxidative stress (OS). Many of their residues can be directly oxidised by ROS, thus serving as buffers, protecting DNA in cells and avoiding irreparable damage. Redox-proteomics are used to study redox-based changes within the proteome[10] enabling a direct detection of lesions caused by the toxicant and of responses at the protein level.

Cysteine, tyrosine and methionine are particularly sensitive to oxidation and their modifications are often measured in redox-proteomics.[11] Protein carbonyls are the most prevalent oxidative lesion of proteins[12] and there is growing evidence of their involvement in signal transduction.[13] They are also increasingly used as biomarkers of oxidative stress in ecotoxicological studies.[14,15]

Despite their advantages, proteomics tools face a major challenge in ecotoxicology as the lack of genomic data for many species renders the identification of particular proteins affected by treatments difficult.[9] In this context, daphnids are a prime choice as the genome of *Daphnia pulex* has recently been sequenced,[16] opening the door to the application of the omics toolbox for this group of organisms. Already among the most widely-studied organisms in ecotoxicology, daphnids are routinely used to assess the toxicity of effluents and novel compounds. As the subject of many official protocols,[17–19] they are an excellent model to better understand NP toxicity to the aquatic environment.

In order to better understand AgNP toxicity to aquatic organisms, this study applied a redox-proteomics approach to *D. magna*. Organisms were exposed to citrate-coated AgNP and toxicity was evaluated using a whole-organism approach and biochemical measurements. In addition to classical biomarkers, we apply for the first time the toolbox of redox-proteomics to this model organism by labelling protein carbonyls with fluorescein-5-thiosemicarbazide (FTC) prior to two-dimensional electrophoresis (2DE).

2 Results

2.1 Characterisation of AgNPs

Fig. S1† shows a low magnification transmission electron microscope (TEM) image of the AgNPs prepared by room temperature reduction of $AgNO_3$ by $NaBH_4$ in the presence of sodium citrate as a stabiliser. The images show that the AgNPs possess a bimodal size distribution, with approximately equal numbers of both sizes. Insets to Fig. S1† show histograms of NP diameters, determined by analysis of TEM images of 300 AgNPs of each size, all located at random locations on the grid. Fitting the histogram to a Gaussian model yielded a mean diameter for the smaller AgNPs of 3.0 ± 0.5 nm, while the larger AgNPs were more polydisperse, with a mean diameter of 11.4 ± 2.2 nm.

High-resolution TEM (HR-TEM) imaging was used in conjunction with selective area electron diffraction (SAED) to confirm the crystallinity and establish the crystal phase of the NPs; see Fig. S2(a) and (b)†. HR-TEM imaging (Fig. 2(a)) showed that the AgNPs are highly crystalline; the lattice fringes shown in Fig. S2(a)† correspond to a d spacing of 2.36 Å, closely matching the (111) spacing reported for the ($Fm3m$) face-centred cubic lattice of silver. SAED patterns of the AgNPs (Fig. S2(b)†) showed reflections that could be indexed to 2.36 Å (111), 2.04 Å (200), 1.45 Å (220), 1.18 Å (222) and 1.02 Å (400), confirming AgNP crystallinity.

Fig. S3† shows the energy dispersive X-ray (EDS) spectrum of the AgNPs, where the Ag peak corresponding to the presence of the NPs is evident. Other elemental peaks assigned to Cu are due to the carbon-coated copper grid support. UV-Vis absorption spectra of the freshly-prepared AgNPs exhibited a strong peak centred at 392 nm, with an absorbance (A_{392}) of 0.859 (Fig. S4†). In comparison, A_{392} for the SAgNP was less than 0.008, corresponding to a > 100-fold decrease in the concentration of AgNPs following ultracentrifugation. The red-shift in the residual absorption band to 407 nm is probably due to the presence of AgNP aggregates that were temporarily re-suspended while extracting the supernatant from the centrifuge tube.

2.2 Immobilisation assay

The impact of AgNP, silver nitrate as well as the supernatant of AgNP was measured as EC50 of immobilisation on *Daphnia magna* neonates according to established OECD[17] protocols. Results (Table 1) show a marked difference in toxicity between AgNPs (47.2 μg L^{-1}) and silver ions (4.5 μg L^{-1}). Additionally, when particles were removed from the AgNP solution by ultracentrifugation, toxicity decreased by a factor of 6.3. Ageing of the particles in the stock solution in the dark did not significantly affect their toxicity.

2.3 Molecular biomarkers

Enzymatic assays did not show statistically significant effects of exposure to either $AgNO_3$ or AgNP (data not shown) for concentrations of up to half their respective EC50. In comparison, relative FTC fluorescence significantly increased when *D. magna* were exposed to 20 μg AgNP per L (54% increase, $p < 0.001$) but was not affected by $AgNO_3$ exposure (Fig. 1). A trend towards an increase of IAF labelling with increasing concentrations of either AgNP or $AgNO_3$ was also observed (Fig. 1), although this increase was only statistically significant at an intermediate concentration of AgNP (15 μg AgNPs per L, 62% increase, $p < 0.01$) and at the highest [Ag^+] (2.5 μg L^{-1} Ag, 63% increase, $p < 0.01$).

Table 1 EC50 for the immobilisation of *Daphnia magna* by silver compounds

Compound	EC50 (μg L^{-1})	95% CI
Fresh AgNP	47.2	43.2–50.5
SAgNP[a]	298.6	274.2–325.1
Ag^+	4.5	2.7–7.4
6 m.o. AgNP[b]	50.2	39.4–64.0
10 m.o. AgNP[b]	50.2	45.2–55.8
10 m.o. SAgNP[a,b]	429.9	376.1–491.3

[a] Supernatant obtained by ultracentrifugation of the AgNP stock, toxicity is in nominal [Ag] pre-centrifugation. [b] Toxicity of AgNP was tested after 6 and 10 months of ageing, and after 10 months for the supernatant.

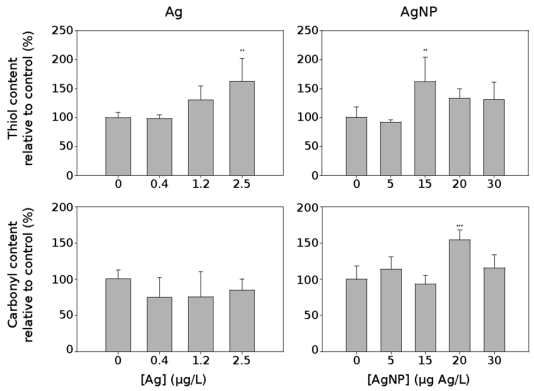

Fig. 1 Measurement of protein carbonyl and thiol content using fluorescent labels on samples exposed to silver nitrate and AgNP: fluorescent values were normalised upon total protein stain to obtain relative fluorescence. (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

2.4 2DE

On 2DE analysis of proteins from samples exposed to AgNP, a total of 10 unique features showed significant volume change of at least 1.5-fold, while 7 were significantly altered in silver nitrate-exposed samples, either at the protein or FTC labelling level (features are shown in Fig. 2). With increasing [AgNP], five features showed an increase of volume of protein stain, one showed decreasing protein stain volumes, two features showed increased carbonyl stain volumes and four showed decreasing carbonyl volumes. Two features (5 and 9) showed significant volume changes both for coomassie stain and FTC labelling. Under increasing [Ag^+], one feature showed an increase in protein stain volume and one a decrease, while one showed increasing carbonyl stain volumes, one a decreasing carbonyl stain volume and three showed a variable response with concentration (*e.g.*: decrease followed by an increase).

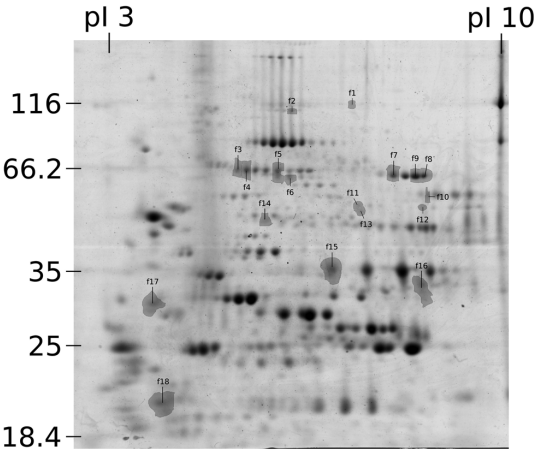

Fig. 2 2DE separation of 100 µg of *Daphnia magna* proteins showing features modified ($p < 0.05$ and >1.5-fold change) by AgNP or $AgNO_3$ exposure.

2.5 Protein identification

Of the 17 features of interest revealed by 2DE, eight were successfully identified by MS/MS analysis (Table 2). Among these, four different hypothetical proteins from the recent *Daphnia pulex* genome[16] were found. In order to gain insight into the potential function of these proteins, bioinformatics tools were used to find similar proteins and the families to which those proteins could be related. The results of these analyses are shown in Table 3. The three bioinformatic tools used (blastp, DELTA-BLAST and InterproScan) were in good agreement as to the type of protein identified here. For brevity, only the best results from the blastp search and the families and main domains found in InterproScan are included in Table 3.

Among the eight identified features, four were annotated as vitellogenin fused with superoxide dismutase (SOD), two as a vitellogenin-like protein, one as hemoglobin and one as the regulatory protein 14-3-3 epsilon. The fold change of the feature volumes from which the above proteins were identified is shown in Table 4 while the features are shown on the reference image of the analysis (Fig. 2).

3 Discussion

3.1 Whole organism toxicity

Results obtained from the immobilisation assay show a clear difference in toxicity between silver nitrate and nanoparticulate silver, with the latter appearing to be 10 times less toxic (considering Ag concentration). As many studies relate AgNP toxicity to their slow dissolution we removed the nanoparticles from solution by ultracentrifugation to determine whether the observed toxicity was a product of dissolved silver, as shown by Kittler *et al.*,[22] or if the particles were responsible for the observed toxicity. As the supernatant was 6.3 times less toxic (as measured by EC50) than the nanoparticle suspension, the observed toxicity of the AgNP is thus associated with the particles themselves and their behaviour in the medium and in contact with the organisms. The present experiment does not control for dissolution of the nanoparticles in the test medium, but the results below indicate that the nanoparticles have a different toxicity mechanism than silver nitrate.

3.2 Biomarkers

Surprisingly few effects were observed with classical biomarkers, even though exposure concentrations went as high as half the EC50 and mortality (less than 20%) was observed at the highest concentrations. Few studies have looked at the impact of AgNP or Ag^+ on daphnid enzymatic activities, but the lack of observed effects of Ag^+ may be linked to the fact that disturbances in osmoregulation are the main mechanism of toxicity of silver ions in these organisms.[23] In addition, Poynton *et al.*[8] did not find an impact of Ag^+ or AgNP on GST mRNA levels.

Although the measured enzymatic activities were not affected by the treatments, the thiol content of proteins from daphnids

Table 2 Identified proteins using LC-MS/MS

Feature	Protein	Organism	Accession number	MW (kDa) (measured/ predicted)	Protein score[a]/ protein C.I. (%)	Total ion score/ total ion C.I. (%)	Sequence coverage (%)	Number of peptides[b]
f1	Hypothetical protein DAPPUDRAFT_318420	*Daphnia pulex*	gi|321469729	98/171	362/100	289/100	17	3
f3	Hypothetical protein DAPPUDRAFT_213992	*Daphnia pulex*	gi|321465380	68/228	153/100	153/100	10	2
f4	Hypothetical protein DAPPUDRAFT_213992	*Daphnia pulex*	gi|321465380	68/228	162/100	144/100	11	2
f5	Hypothetical protein DAPPUDRAFT_213992	*Daphnia pulex*	gi|321465380	68/228	205/100	162/100	16	2
f7	Hemoglobin	*Daphnia magna*	gi|2105139	66/38	319/100	301/100	19	2
f10	Hypothetical protein DAPPUDRAFT_313764	*Daphnia pulex*	gi|321474716	16/170	283/100	283/100	12	2
f15	Vitellogenin fused with superoxide dismutase	*Daphnia magna*	gi|39979307	40/225	559/100	485/100	14	2
f18	Hypothetical protein DAPPUDRAFT_326737	*Daphnia pulex*	gi|321460806	19/29	447/100	381/100	52	4

[a] The protein score probability limit (where $p < 0.05$) is 86. [b] Number of MS/MS patterns assigned to peptides with confidence interval above 95%.

was increased by $AgNO_3$ and AgNP (Fig. 1). This may represent an attempt by the animals to decrease the toxicity of silver ions (which are also likely to be present in small concentrations in the AgNP exposures) as thiols are known to react with silver ions.[24] The increase of the thiol content following AgNP exposures is not expected when the usual link between AgNPs and OS is considered. Protein thiols are easily oxidised when the redox balance of the cell is disturbed, as they act both as buffers and sensors of oxidative stress in cells. The observed increase, if not linked to silver dissolved from the AgNPs, may be a result of the cell restoring its redox balance to counteract the stress of exposure. This is reinforced by the fact that there is an increase in the carbonyl content of the proteins in daphnids exposed to AgNPs but not silver nitrate (Fig. 1). Protein carbonyls are the main lesion found in proteins, as they are generated by the oxidation of various amino acids.[25] The increase in protein carbonyls indicates that some level of oxidative stress was caused by the AgNP, although not enough to also oxidise the protein thiols. In contrast, the lack of change of the level of protein carbonyls in the exposures to silver nitrate suggests that significant OS did not occur in the daphnids, perhaps as a result of the increasing thiol content with increasing silver concentration.

3.3 Proteomics approach

The number of features evident in 2DE separations as significantly affected by both treatments was similar (10 and 7), and no feature was commonly modified by both treatments. This

Table 3 Blast and interproscan search of hypothetical proteins

Feature	Accession number	Best hit[a] (protein (*species*), *E*-value, identity %)	Families and domains[b]	Go functions[b]
f1	gi|321469729	Vitellogenin-like protein (*Lepeophtheirus salmonis*) *E*: 3×10^{-106}, identity: 24%	von Willebrand factor, type D domain; vitellinogen, superhelical; vitellinogen, beta-sheet N-terminal	Lipid transport activity; lipid transport
f3, f4, f5	gi|321465380	Vitellogenin fused with superoxide dismutase (*Daphnia magna*) *E*: 0.0, identity: 52%	Superoxide dismutase (Cu/Zn)/chaperones; von Willebrand factor, type D domain; vitellinogen, open beta-sheet; vitellinogen, beta-sheet N-terminal	Superoxide metabolic process; metal ion binding; oxidation-reduction process; lipid transport activity; lipid transport
f10	gi|321474716	Vitellogenin-like protein (*Lepeophtheirus salmonis*) *E*: 3×10^{-106}, identity: 24%	von Willebrand factor, type D domain; vitellinogen, superhelical; vitellinogen, beta-sheet N-terminal	Lipid transport activity; lipid transport
f18	gi|321460806	14-3-3 Protein epsilon (*Schistocerca gregaria*) *E*: 2×10^{-170}, identity: 91%	14-3-3 protein	protein domain specific binding

[a] Best hit results are obtained using a blastp search.[20] [b] Families, domains and GO functions were obtained using Interproscan.[21]

Table 4 Fold change of feature volumes of identified proteins[a]

	AgNP (coomassie/fluorescence)				Ag^+ (coomassie/fluorescence)		
Protein (feature)	5 ppb	15 ppb	20 ppb	30 ppb	0.4 ppb	1.2 ppb	2.5 ppb
Vitellogenin-like protein (f1)	—/—	—/—	—/—	—/—	—/−1.3	—/−1.7	—/−1.1
Vitellogenin fused with superoxide dismutase (f3)	—/—	—/—	—/—	—/—	−1.1/—	1.1/—	1.8/—
Vitellogenin fused with superoxide dismutase (f4)	1.1/—	1.5/—	1.4/—	1.5/—	—/—	—/—	—/—
Vitellogenin fused with superoxide dismutase (f5)	1.1/−1.1	−1.1/−1.4	−1.6/−1.7	−1.4/−1.6	—/—	—/—	—/—
Hemoglobin (f7)	1.0/—	1.4/—	1.3/—	1.6/—	—/—	—/—	—/—
Vitellogenin-like protein (f10)	—/—	—/—	—/—	—/—	—/−1.4	—/1.0	—/1.4
Vitellogenin fused with superoxide dismutase (f15)	—/−1.2	—/−1.5	—/−1.8	—/−1.7	—/—	—/—	—/—
14-3-3 Protein epsilon (f18)	—/—	—/—	—/—	—/—	—/−1.1	—/−2.0	—/−2.0

[a] Fold changes are relative to control and given only for treatment where the feature was significantly changed (ANOVA $p < 0.05$).

again suggests different toxicity pathways or mechanisms between the two forms of silver tested. When comparing the results from the protein-stained and FTC-labelled gels though, two of the modified features (f5 and f9) showed changes at the protein and carbonyl content level. This most likely means that they both contained a protein whose expression was modified, rather than carbonylation as, in both cases, the change in protein and carbonyl stain volume followed the same trend with similar fold-changes. In the case of features where a change was observed only for carbonyls or protein abundance, it is likely one was modified and not the other. More in depth analysis is required for those features in order to determine the exact level of carbonylation of the proteins concerned.

Identification of the features led to the realisation that a lot of the proteins modified showed overlap between the two compounds, as two features modified by ionic silver annotated as a vitellogenin-like protein (f1 and f10) and one as a vitellogenin fused with SOD (f3); while three features modified by AgNP contained a protein annotated as a vitellogenin fused with SOD (f4, f5 and f15). This strong overlap between the two treatments is partly a result of some features being part of the same "train" (a chain of features showing similar shape and MW, but slightly different pI). As a matter of fact, features 3, 4 and 5 were identified as the same protein and are part of the same train. It should be noted that, in the case of those features, the repetition may be due to the extensive maturation process of vitellogenins (see below).

3.4 Role of modified proteins

Mass spectrometric analysis of the significant features led to the identification of two proteins from *D. magna*: hemoglobin (f7) and vitellogenin fused with SOD (f15). All other features that yielded results from this analysis were identified as hypothetical proteins from the recently completed *D. pulex* genome (Table 2).[16] In order to gain insight into the role of those proteins, blastp,[20] DELTA-BLAST[26] and InterproScan[21] were used to find sequence similarity with known proteins or protein domains. In all cases, a clear result was obtained, with the three search engines yielding similar results and agreeing on the likely function of the hypothetical protein. Table 3 shows the result of this analysis. As features 3, 4 and 5 contain the same protein, three different functions were found for the hypothetical proteins. Features 1 and 10 appear to be vitellogenin-like proteins (although of different mass and pI), features 3, 4 and 5 are a vitellogenin fused with SOD and feature 18 has high sequence identity with members of the 14-3-3 protein family of regulatory proteins.

Overall, six identified features out of eight appear to be either vitellogenin-like or vitellogenin fused with SOD. At present, studies have shown the presence of two isoforms of vitellogenin fused with SOD in *D. magna*, both of which were also found here. DmagVTG1[27] was identified from feature 15, while DmagVTG2[28] was found in features 3, 4 and 5. A literature search did not enable the finding of other vitellogenins in *D. magna*, but two of the hypothetical proteins appear to be vitellogenin-like proteins. This would not be surprising considering the high rate of gene duplication found in the *D. pulex* genome.[16]

In oviparous species, vitellogenins are known to be differentially regulated at the gene level under many stress circumstances, and normally undergo major PTM due to their involvement in the egg maturation process.[27] In the case of daphnids, this is also true during parthenogenetic reproduction, as the main product of DmagVTG1 is gradually cleaved during egg maturation.[27] There is no known direct association of vitellogenin expression in crustaceans to a particular stressor-type or environmental factor, in contrast to fish where it is related to oestrogenic compounds when found in males.[29] Yet work has been completed to establish vitellogenins as an easily applicable biomarker between invertebrate species, including daphnids.[30] In correspondence to this, vitellogenin has been proposed as a marker of general stress in the zebra fish, especially when egg or embryo development is followed rather than adults.[31] Unsurprisingly, all the features from which vitellogenins were identified here showed much lower masses than predicted from the whole-protein sequence. This is consistent with their function and with previous results from Kato *et al.*,[27] who showed that the 220 kDa protein DmagVTG1 was gradually cleaved into different proteins of smaller size. As vitellogenin maturation is thought to

be a highly regulated process affected by stressors and development stages,[31,32] variations in the maturation process could be used as indicators of toxic stress. Gel-based proteomics would thus be an interesting method to study differences in cleavage-site and PTMs (*i.e.* phosphorylation, glycolysation, lipidation) of vitellogenins in daphnids.

Another point of interest concerning this protein is that vitellogenins fused with SOD were only found in daphnids[28] and in *Artemia*.[32] The presence of a SOD domain may indicate a link with oxidative stress. Hannas *et al.*[29] found that many of the stressors that upregulate DmagVTG2 are also linked to OS while Kato *et al.*[27] showed that the SOD domains present some, although weak, SOD activity. Since oxidative stress is a likely candidate for the toxicity mechanism of AgNP, this could explain the impact of those compounds on vitellogenins.

Exposure to AgNP led to an increase in hemoglobin, the main oxygen carrier in daphnids. Hemoglobin expression is known to be highly variable in daphnids, with its levels increasing when oxygen levels are low or temperature changes rapidly,[33,34] and in response to toxic stress.[35] In both cases, an increase in ROS may result in increased hemoglobin expression, as in *D. magna*, hemoglobin expression is under the control of hypoxia-inducible-factor-1 (HIF-1).[34] The observed increase is thus a likely sign of OS, as oxygenation was maintained throughout the 24 h exposures. This also agrees with the observed increase in protein carbonylation discussed above.

Exposure to silver nitrate led to a 2-fold decrease in the level of carbonylation of a likely member of the 14-3-3 protein family. Although no studies have been published on 14-3-3 proteins in daphnids, this family of proteins is well-studied elsewhere. They have diverse roles including control of the cell cycle and apoptosis, signal transduction and regulation of the subcellular localisation of proteins. They are found in all eukaryotes.[36] In shrimps, they have been shown to be up-regulated during viral infection[37] and microbial challenge.[38] The fact that the 14-3-3 protein observed here showed a lower level of carbonylation following ionic silver exposure as well as a mass almost half that expected from the gene sequence may either indicate proteolytic cleavage of the protein (with loss of carbonylation) or a higher turnover rate, both potentially leading to changes in cell-signalling following exposure.

Globally, the response from the two forms of silver shown here is quite distinct. Although no clear conclusion can be drawn concerning the mechanism of action of AgNP, its effect seems to be quite different from that of $AgNO_3$. With the exception of features 3 and 4 (both containing DmagVTG2 and increasing in volume with exposure concentration), all features were modified by only one of the two forms of silver tested. In addition to the fact that toxicity of the AgNP preparation was strongly reduced following centrifugation, we propose that the particles present a toxicity different from that of silver ions, albeit at a lower level. This is in contradiction to previous studies and the general understanding that a major portion of AgNP toxicity originates from their dissolution.[22] Yet this is in agreement with other studies in daphnids where particles were found to be less toxic than silver nitrate[7] and where particle toxicity did not follow the same mechanism as silver nitrate toxicity.[8] Although our results do not give clear insight into the mechanism of toxicity of AgNP, the difference observed between the effects of AgNP and Ag^+ at the molecular level indicate that the two species of silver affect daphnids differently. Whether this is an effect of whole particles acting directly at the cellular level as is known for metal NPs of similar sizes or from the particles acting as "delivery" vehicles for ionic silver to specific parts of the daphnid cannot be concluded from the present study.

4 Experimental

4.1 Chemicals

The protein assay dye reagent concentrate was obtained from Bio-Rad (CA, USA). Unstained protein molecular weight markers for SDS-PAGE were from Thermo Scientific (Rockford, IL, USA). Immobiline drystrips and IPG buffer were obtained from GE Healthcare (UK). All other chemicals were sourced from Sigma-Aldrich Ireland Ltd. (Arklow, Co. Wicklow) and used as received.

4.2 Synthesis and characterisation of AgNPs

Nanopure H_2O (18.2 MΩ cm), purified using an Elgastat Prima purification system, was employed during all experiments. All synthetic glassware was first cleaned with aqua regia (3 HCl : 1 HNO_3), and then thoroughly rinsed with deionised water. In a typical synthesis, 12 mL of a 0.2% solution of silver nitrate was added to 488 mL of deionized water and heated to 100 °C. 11.6 mL of a 1% solution of sodium citrate in deionised water was added, followed 30 s later by the quick injection of 5.5 mL of a freshly prepared, ice-cold solution of 0.038 g sodium borohydride and 0.5 g sodium citrate in 50 mL deionised water. The solution was stirred for two minutes, after which it was cooled to room temperature, and stored in the dark.

UV-Vis absorption spectra were recorded using a Shimadzu UV PC-2401 spectrophotometer equipped with a 60 mm integrating sphere (ISR-240A, Shimadzu). Spectra were recorded at room temperature using a quartz cuvette (1 cm) and corrected for the solvent absorption. AgNP and SAgNP samples were diluted 1 : 5 in deionised water for spectroscopic comparison. TEM images and SAED patterns were acquired using a high-resolution JEOL 2100 electron microscope, equipped with a LaB_6 thermionic emission filament and Gatan DualVision 600 Charge-Coupled Device (CCD), operating at an accelerating voltage of 200 keV. EDS were recorded using an Oxford INCA x-sight detection spectrometer. Spectra were obtained from an area of the grid where there was a large amount of NPs. A process time of 3–4 seconds was used and the spectra obtained using an integration time of 40 s. TEM samples were prepared by depositing a small aliquot of the AgNPs onto a carbon-coated copper grid (Agar Scientific), which was allowed to evaporate under ambient conditions. Data for size distribution histograms were acquired by analysis of TEM images of NPs randomly located at different regions of the grid. NP diameter was determined by manual inspection of the digital images; in the case of anisotropic structures, the diameter was determined using the longest axis.

4.3 AgNP supernatant

To remove particles from solution, the AgNP solution was centrifuged at 100 000*g* for 30 min. Supernatants were then used in the same way as the particle suspension. In order to better illustrate the impact of particle removal on toxicity, supernatant "concentration" is noted in [Ag] equivalents: the nominal concentration of Ag the solution would contain if particles had not been removed. Before use, supernatants were kept in the dark at room temperature.

4.4 *Daphnia magna* culture

A clonal culture of *D. magna* was maintained in Elendt M4 medium[39] in plastic aquaria at 20 ± 1 °C with a 16 : 8 light–dark cycle and constant bubbling. *D. magna* were fed live *Chlorella vulgaris* to a concentration of 50 000 cells per mL at least 4 times per week. Brood stocks were maintained at 50 females per 4 L. Half the medium was renewed two or three times per week. Neonates were removed at least three times per week. Prior to exposures, neonates were kept for 7 days (in 10 mL of medium per neonate), with daily feed of *C. vulgaris*, in order to obtain enough tissue for biochemical assays.

4.5 Immobilisation assay

All assays were performed according to OECD[17] recommendations. At least 30 neonates were exposed, in 4 mL M4 per neonate, in glass beakers at 20 ± 1 °C under a 16 : 8 light–dark cycle. Immobilisation was observed after 24 and 48 hours. Results were fitted with a two-parameter log-logistic model using the dcr package in R[40,41] in order to estimate EC50 values. Unless stated otherwise, assay to AgNP and their supernatants were conducted within a week of AgNP synthesis.

4.6 Exposures

All exposures for biochemical measurements were performed for 24 h using 7 days old *D. magna* in 1 L glass beakers at 100 daphnids per beaker in the same conditions as culture. Daphnids were exposed to four concentrations of AgNP (5, 15, 20 and 30 μg Ag L^{-1}) and three concentrations of $AgNO_3$ (0.4; 1.2 and 2.5 μg Ag L^{-1}). All exposures were repeated four times and control exposures were ran in parallel. Exposures to AgNP were conducted within a week of synthesis. At the end of the exposure, live daphnids were sieved and blotted dry before transfer to a microcentrifuge tube and flash freezing in liquid N_2, they were then kept at −80 °C until homogenisation.

4.7 Homogenisation and enzymatic assays

Nitrogen bubbled homogenisation buffer (10 mM Tris–HCl, pH 7.2, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA and 1 mM PMSF) was added to frozen daphnids (300 μL for one hundred 7 days old daphnids) in a glass-teflon homogeniser, prior to motor-driven homogenisation for one minute. Homogenates were then centrifuged at 14 000*g* for 1 h, pellets were discarded and supernatants were aliquoted for biochemical assays. These were either performed on the day of homogenisation or aliquots were frozen at −80 °C immediately.

Protein concentrations were assessed by a Bradford assay[42] in microtiter plates as per manufacturer's instructions (Bio-Rad Protein Assay Dye Reagent).

Catalase assays were performed according to the method of Beers and Sizer.[43] Briefly, 5 μg protein was added to 16.7 mM phosphate buffer, pH 7.0, containing 19.7 mM hydrogen peroxide. A_{240} was measured immediately for 3 minutes using a dual-beam spectrophotometer and a quartz cuvette. One unit of activity was defined as the decomposition of one micromole of peroxide per minute in the assay conditions used. Glutathione transferase activity was assessed using the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione, according to Habig *et al.*[44] using a protocol modified for microtiter plates. Briefly, 8 μg protein was added to a final reaction mixture of 75 mM phosphate buffer, pH 6.5, 1 mM CDNB and 5 mM reduced glutathione (added last to initiate the reaction). Activity was followed by the increase of A_{340} and one unit is defined as the production of one micromole of conjugate per minute. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was measured in microtiter plates by following the reduction of NAD in the presence of glyceraldehyde-3-phosphate.[45] Five μg protein was added to a reaction mixture containing 15 mM sodium pyrophosphate buffer, pH 8.5, 30 mM sodium arsenate, 0.25 mM NAD, 3.25 mM dithiothreitol and 12.5 mM DL-glyceraldehyde-3-phosphate (added last to initiate the reaction). Activity was followed as an increase in A_{340} and one unit is defined as the reduction of one micromole of NAD per minute.

4.8 PTM labelling and one-dimensional electrophoresis

In order to estimate protein thiol content, 100 μg protein was labelled with 5-(iodoacetamido)fluorescein (IAF). IAF was added to homogenates from a 20 mM stock in DMSO to a final concentration of 0.2 mM, and incubated at 4 °C for 2 h in the dark.[46] Proteins were then precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10% w/v, incubated on ice for 5 min and centrifuged at 11 000*g* for 3 min. The pellet was resuspended in 40 μL of water, and 500 μL of acetone was added to remove unbound IAF. Samples were then kept at −20 °C for at least one hour. Before electrophoresis, proteins were centrifuged at 11 000*g* for 3 min, acetone was removed, and pellets were dried in the dark. Sample buffer was added to solubilise the proteins and those were run on 10% polyacrylamide gels, at a loading of 20 μg per lane, with four replicate lanes per sample, as per Laemmli.[47] After electrophoresis, gels were scanned using a Typhoon scanner, model 9410 (Amersham Biosciences), with an excitation wavelength of 488 nm and emission light of 520 ± 20 nm (bandpass filter). After acquisition of the fluorescence image, gels were stained with colloidal coomassie,[48] and gel images were acquired with a GS-800 Calibrated Densitometer (BioRad, Hercules, CA, USA).

Protein carbonyls were labelled by adding (FTC) to tissue homogenates (100 μg) to a final concentration of 1 mM (adapted from Chaudhuri *et al.*[49]). Samples were incubated for 2 h in the dark at 4 °C before precipitation of proteins with a final concentration of 10% w/v of TCA. Pellets were washed twice with 500 μL of ice cold 1 : 1 ethanol–ethylacetate. Prior to resuspension and electrophoresis as above, pellets were

centrifuged and dried to make sure no solvent remained in the samples. Gels containing FTC labelled samples were scanned for fluorescence and protein content as described above.

4.9 Two-dimensional electrophoresis and protein identification

Aliquots of freshly prepared homogenates containing 500 μg protein were labelled with FTC as above, with the exception that rehydration buffer (5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% IPG buffer) was used to resuspend the pellets. Of this, 125 μL, containing 125 μg protein, were loaded unto an Immobiline DryStrip (pH 3-10 NL, 7 cm, GE Healthcare), which was rehydrated overnight in the dark. Isoelectric focusing was performed on a PROTEAN IEF system (Bio-Rad), according to the strip manufacturer's recommendation. Strips were reduced in equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% w/v SDS, 20% v/v glycerol) containing 2% w/v DTT for 20 min and thiols were then blocked with equilibration buffer containing 2.5% w/v iodoacetamide for 20 min. After focusing, strips were loaded unto 10% polyacrylamide gels for SDS-PAGE separation. Gels were scanned for fluorescence and then stained with colloidal coomassie as for 1DE. Image analysis was performed using the Progenesis SameSpots software (Nonlinear Dynamics Limited, UK). Experiments were defined by compound, with the exposure concentrations representing treatments. Spots were considered of interest when showing a 1.5-fold change between treatments as well as having a $p < 0.05$ in ANOVA. Significant, well resolved spots of sufficient intensity were then selected for mass spectrometric analysis.

Selected spots were excised manually using clean pipette tips and in-gel digested with trypsin according to Almeida *et al.*[50] Extracted peptides were loaded onto a R2 micro column (RP-C18 equivalent) where they were desalted, concentrated and eluted directly onto a MALDI plate using α-cyano-4-hydroxycinnamic acid as the matrix solution in 50% acetonitrile and 5% formic acid. Mass spectra of the peptides were acquired with positive reflectron MS and MS/MS modes using a MALDI-TOF/TOF MS instrument (4800 plus MALDI TOF/TOF analyzer) with exclusion list of the trypsin autolysis peaks (842.51, 1045.56, 2211.11 and 2225.12). The collected MS and MS/MS spectra were analysed in combined mode by using the Mascot search engine (version 2.2; Matrix Science, Boston, MA) and the NCBI database restricted to 50 ppm peptide mass tolerance for the parent ions, an error of 0.3 Da for the fragments, one missed cleavage in peptide masses, and carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. No taxonomy restrictions were applied. The identified proteins were only considered if a MASCOT score above 95% confidence was obtained ($p < 0.05$) and at least one peptide was identified with a score above 95% confidence ($p < 0.05$). This analysis was conducted by the Analytical Services Unit, Instituto de Tecnologia Química e Biológica (ITQB), New University of Lisbon, Lisbon, Portugal.

4.10 Data analysis

Images from 1DE were analysed with the Quantity One software (Bio-Rad) to obtain one trace measurement per lane. Fluorescence values were normalised for loading by dividing them with the trace coomassie value for the same lane. Statistical analysis of enzymatic and PTM assays were performed by one-way ANOVA with a Holm–Sidak post-hoc test (*versus* control), using the Sigmaplot 10.0 software (Systat Software, Inc.).

4.11 Hypothetical proteins

Hypothetical proteins (HP) identified by MS/MS were studied using bioinformatics tools to gain insight into their possible biological functions. This was achieved using blastp[20] and DELTA-BLAST[26] from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sequence similarity with known proteins. In addition, conserved sites, domains and families present in the HP were studied using the Interproscan tool[21] from the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Results include the highest-scoring, non-hypothetical, protein from blastp, as well as the domains and families identified from Interproscan. In the present study, those were always in accordance with results from DELTA-BLAST.

5 Conclusions

This study compared the toxicity of citrate-coated AgNP and silver nitrate to *D. magna* at the organism level, using biochemical biomarkers and with redox-proteomic tools. AgNP were found to be about ten times less toxic than silver nitrate. Although measured enzymatic activities (GST, catalase and GAPDH) were not affected by the treatments, an increase of protein carbonylation was observed following AgNP exposure, indicating OS, while no sign of OS was found for $AgNO_3$ exposure. Proteins identified following 2DE separation showed signs of general stress, with most of the features modified by the treatments containing vitellogenins, indicating that the maturation process or expression of vitellogenins is affected, in different ways, by silver nitrate as well as AgNP. Hemoglobin was also increased by AgNP treatment, which may be linked to disruption of cellular respiration.

Overall, different molecular responses were found for the two forms of silver. Although more studies are required to better understand AgNP toxicity, the lower toxicity of AgNP often reported relative to silver ions[6,7] does not warrant its relative innocuity, as different toxicity mechanisms may mean different toxic interactions or lead to population effects that cannot be predicted by the relative EC50 of the two forms of silver.

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Proteomic evaluation of citrate-coated silver nanoparticles toxicity in *Daphnia magna*

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Supplementary Figures

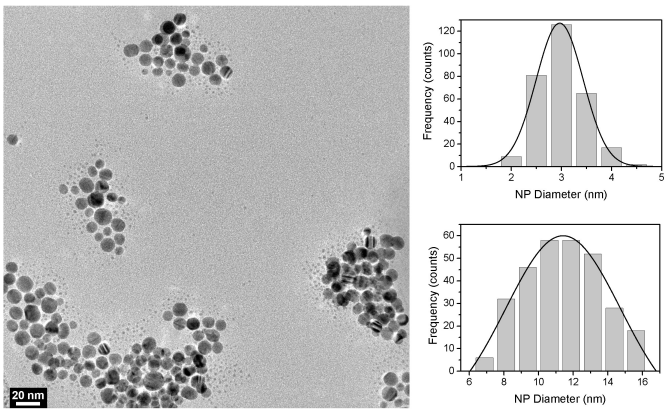

Figure S.1. TEM images of citrate-stabilised AgNPs, showing a bimodal size distribution. Inset: Size histograms of both sizes of AgNPs with Gaussian curves fitted to the data.

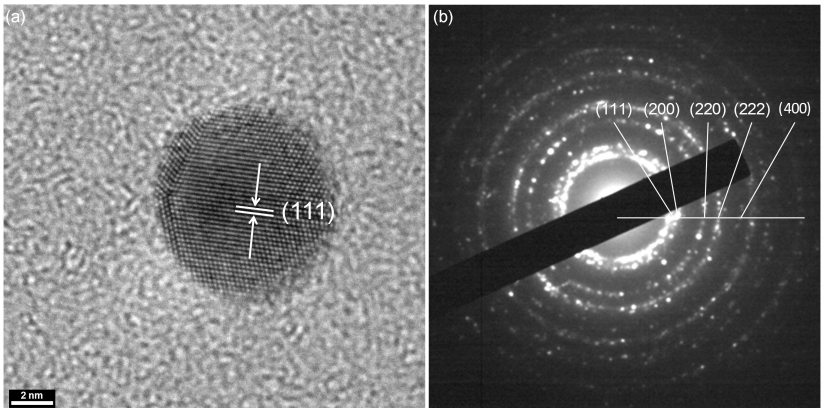

Figure S.2. (a) HR-TEM image of a single AgNP, showing an interplanar spacing of 2.34 Å, corresponding to the (111) interplanar spacing. (b) SAED pattern with indicated reflections characteristic of crystalline silver.

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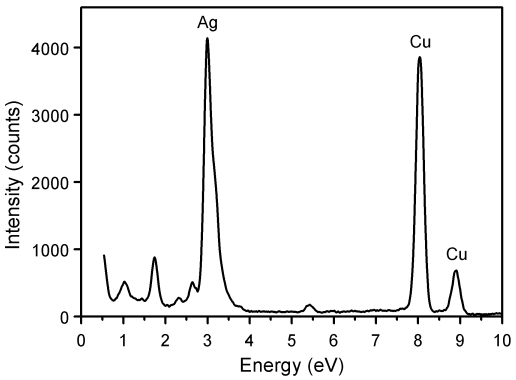

Figure S.3. EDS spectrum of citrate-stabilised AgNPs.

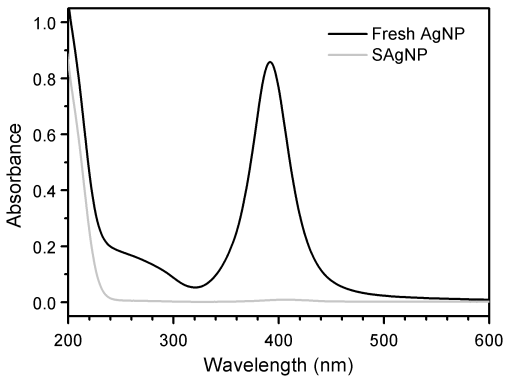

Figure S.4. UV-Vis absorption spectrum of an aqueous dispersion of the freshly prepared AgNP and SAgNP supernatant samples.

Chapter 4

Solid-Phase Hydrazide enrichment of carbonylated proteins

4.1 Introduction

Protein carbonylation is widely used as an indicator of oxidative stress and has been related to ageing (Stadtman, 2006) and many diseases (Sultana et al., 2010). Arising from the direct oxidative modification of amino acids or the conjugation of lipid oxidation products or reducing sugars and their derivatives (Stadtman and Levine, 2000), protein carbonyls present an important structural diversity coupled to a low abundance (Stadtman and Levine, 2000; Madian and Regnier, 2010b). Generally considered irreversible, carbonylation affects enzyme activity and protein stability. There is now evidence that protein carbonylation may also be involved in cell signaling, marking specific proteins for degradation by the proteasome, as observed for annexin A1 in pulmonary artery smooth muscle cells following treatment with endothelin-1 (Wong et al., 2010). There is also recent evidence of the presence of enzymatic mechanisms enabling decarbonylation of

some proteins (Wong et al., 2013).

Traditionally, protein carbonyls are studied through their derivatisation with dinitrophenylhydrazine (DNPH) in acidic conditions. The carbonyl content can then be measured spectrophotometrically, by ELISA or through immunoblot (Fedorova et al., 2013). The validity of this method comes from the relatively high specificity of the reaction of hydrazine with the carbonyl of ketones and aldehydes, chemical groups rarely found in biological macromolecules. Most of the signal measured by DNPH is thus linked to the presence of protein carbonyls. Based on this specificity of hydrazines, and the related hydrazides, many new labels are now used to detect protein carbonyls. These enable the linking of biotin (Madian and Regnier, 2010a), fluorophores (Chaudhuri et al., 2006) or iTRAQ labels (Palmese et al., 2012) to the carbonyls of proteins. As mass spectrometry is the method of choice to detect carbonylation sites, there is a need to develop tags that enable better detection of carbonylation sites, although there is also an interest in using label-free approaches for some forms of protein carbonyl (Guo and Prokai, 2011).

The instability of the hydrazones formed between hydrazines and carbonyls often leads researchers to reduce the hydrazone to an amine in order to ensure a stable labelling of carbonyls (Fedorova et al., 2013). Although problematic for labelling, this reversibility led to the development of a covalent chromatography method for 4-hydroxy-2-nonenal (HNE) modified peptides, enabling purification of carbonyl-containing peptides and recuperation of the unmodified carbonyls (Roe et al., 2007). This approach is based on the use of solid-phase hydrazide (SPH) to trap carbonylated peptides, enabling their enrichment before further analysis by MS.

We here expand the application of SPH to the study of whole proteins modified by metal catalysed oxidation (MCO), thus showing that primary carbonyls can be studied by SPH and not only adducts of lipid oxidation products. This

covalent chromatography is based on the commercially available Affi-Gel® Hz hydrazide beads (Bio-Rad) and relies on the stability of hydrazone bonds at alkaline pH along with their reversibility in acidic conditions, as initially demonstrated for peptides by Roe et al. (2007). We chemically oxidised bovine serum albumin (BSA) by MCO and demonstrate the possibility to enrich and label the purified carbonylated proteins with fluorescein-5-thiosemicarbazide (FTC) prior to electrophoretic analysis.

4.2 Material & Methods

4.2.1 Chemicals

Affi-Gel® Hz hydrazide beads and Protein Assay Dye Reagent concentrate were sourced from Bio-Rad (CA, USA). Guanidine-HCl was from Fisher Scientific (Dublin, Ireland). All other chemicals were bought from Sigma-Aldrich Ireland Ltd. (Wicklow, Ireland).

4.2.2 Metal catalysed oxidation

BSA was oxidised by metal catalysed oxidation (MCO) following the protocol of Maisonneuve et al. (2009). Briefly, ascorbic acid and $FeCl_3$ were added to 10 mg/mL BSA in oxidation buffer (50 mM HEPES, pH 7.4, 100 mM KCl and 10 mM $MgCl_2$). The concentration of ascorbic acid and $FeCl_3$ was varied to obtain different levels of oxidation. Sample 1 contained 25 µM ascorbic acid and 0.1 µM $FeCl_3$, each subsequent sample contained ten times more of the MCO reagents, so that sample 4 contained 25 mM ascorbic acid and 100 µM $FeCl_3$. The samples were then incubated at 37 ℃ for 14 h under gentle agitation. Oxidation was stopped by adding EDTA to a final concentration of 1 mM.

Prior to analysis and isolation, the MCO reagents and EDTA were removed by ultrafiltration using Amicon® Ultra-4 (Millipore) centrifugal filter devices with a

3 kDa molecular weight cut-off. Oxidised protein solution (2 mL) was made up to 5 mL with oxidation buffer, centrigued in a swinging bucket rotor at 4 000 g until the volume was reduced to 0.5 mL and oxidation buffer was added to make up the volume to 5 mL. This was repeated three times to remove the MCO reagents. Proteins were quantified post-oxidation by Bradford assay (Bradford, 1976) as per manufacturer's instructions (Bio-Rad Protein Assay Dye Reagent). Aliquots were immediately labelled for carbonyls before electrophoretic characterisation (see below) and 5 mg protein was added to SPH beads immediately for carbonyl isolation.

4.2.3 Carbonylated protein isolation

In order to isolate carbonylated proteins, 0.5 mL of hydrazide bead slurry per sample (equivalent to 0.3 mL of compacted beads) were washed thrice by adding a large excess of coupling buffer (6 M guanidine-HCl, 100 mM acetate buffer, pH 4), decanting and removing the supernatant. After the final wash, beads were resuspended in coupling buffer to their initial slurry volume. Slurry (0.5 mL) was transferred to 2 mL microcentrifuge tubes, 5 mg oxidised BSA was added and the volume was made up to 2 mL with coupling buffer. Samples were stirred on a rotating shaker at 4 ℃ for 20 h. After coupling, tubes were centrifuged at 11 000 g for 3 min to pellet the beads, the supernatant was removed and 1.5 mL of buffer A (6 M guanidine HCl, 0.4 M ammonium carbonate, pH 8.3) was added. Tubes were inverted to resuspend the beads. The procedure was repeated a total of 6 times with buffer A to remove unbound proteins. To avoid interference from guanidine in future steps and removing any remaining unbound protein, beads were washed 6 times with 1.5 mL of buffer B (20 mM ammonium carbonate, adjusted to pH 8.3 with formic acid or ammonia)

After the last wash, the beads were resuspended in 0.5 mL 10 % (v/v) formic acid and incubated for 2h under gentle agitation at room temperature. Beads

were then centrifuged, the supernatant was retained and another 0.5 mL of formic acid solution was added. Tubes were inverted to resuspend the beads and centrifuged. The supernatant was then pooled with the previous one forming the released fraction. Proteins were quantified as previously and labelled for carbonyl detection (see below).

4.2.4 Relative carbonyl content

Protein carbonyls were labelled by adding FTC to 40 µg oxidised BSA or released proteins to a final concentration of 1 mM (Chaudhuri et al., 2006). Samples were incubated for 2h in the dark at 4 ℃ before precipitation of proteins to remove unbound FTC. Precipitation was performed by addition of trichloroacetic acid (TCA) to a final concentration of 10 % w/v and 0.02 % sodium deoxycholate followed by incubation on ice for 5 min and centrifugation at 11 000 g for 3 min. Pellets were washed twice with 500 µL of ice cold solvent (1:1 ethanol:ethylacetate) and centrifuged at 11 000 g for 3 min before solvent removal and drying. Sample buffer was added to solubilise the proteins and those were then electrophoresed on 12 % polyacrylamide gels as per Laemmli (1970), in non-reducing conditions. Five micrograms were loaded per lane, with four replicates per sample. After electrophoresis, gels were scanned using a Typhoon scanner, model 9410 (Amersham Biosciences), with an excitation wavelength of 488 nm and emission light of 520 ± 20 nm (bandpass filter). After acquisition of the fluorescence image, gels were stained with colloidal coomassie (Dyballa and Metzger, 2009), and images were acquired with a GS-800 Calibrated Densitometer (BioRad, Hercules, CA, USA).

Gel images were analysed using the Quantity One software (Bio-Rad) to obtain a single trace measurement per BSA band. Relative fluorescence values were calculated by dividing the trace fluorescence by the trace coomassie value of the corresponding band.

4.3 Results

4.3.1 Metal catalysed oxidation

Colour was observed in the two most oxidised samples (3 and 4) while the first two samples did not show significant colour. This colour was linked to the proteins as they were found in the retentate after ultrafiltration. Additionally, sample 4 presented a small precipitate when concentrated by ultrafiltration, this precipitation was observed in previous experiments and only found in highly oxidised samples. The precipitate could always be re-solubilised by mixing so that no visible particles were left in the samples. In addition to these macroscopic signs of oxidation, samples were analysed by one-dimensional electrophoresis (1DE) after labelling with FTC to determine the relative carbonyl content of the samples. The 1DE profiles show a clear change as the oxidation level of samples increases (Fig. 4.1 A and B). Sample 1 presents a profile similar to that of the untreated BSA (not shown), with a main band at 48.3 kDa (non-reducing conditions), and a few minor bands of higher mass, with the band of an apparent mass of 84 kDa likely being a multiplet of BSA. The fluorescence image shows a similar profile. Interestingly sample 4 presented many minor bands of lower mass, indicating a breakdown of BSA under greater oxidation, and that the fragments contained carbonyls.

A clear increase in the intensity of the fluorescence bands is observed with increasing levels of oxidation. This was quantified by dividing the fluorescence signal of the BSA band by the coomassie signal, in order to obtain a relative fluorescence. This is known to be related to the carbonyl content of the proteins (Chaudhuri et al., 2006). The carbonyl content increases between samples 1 and 4, as the relative fluorescence goes from 167 to 7075 arb. unit. (Fig. 4.2).

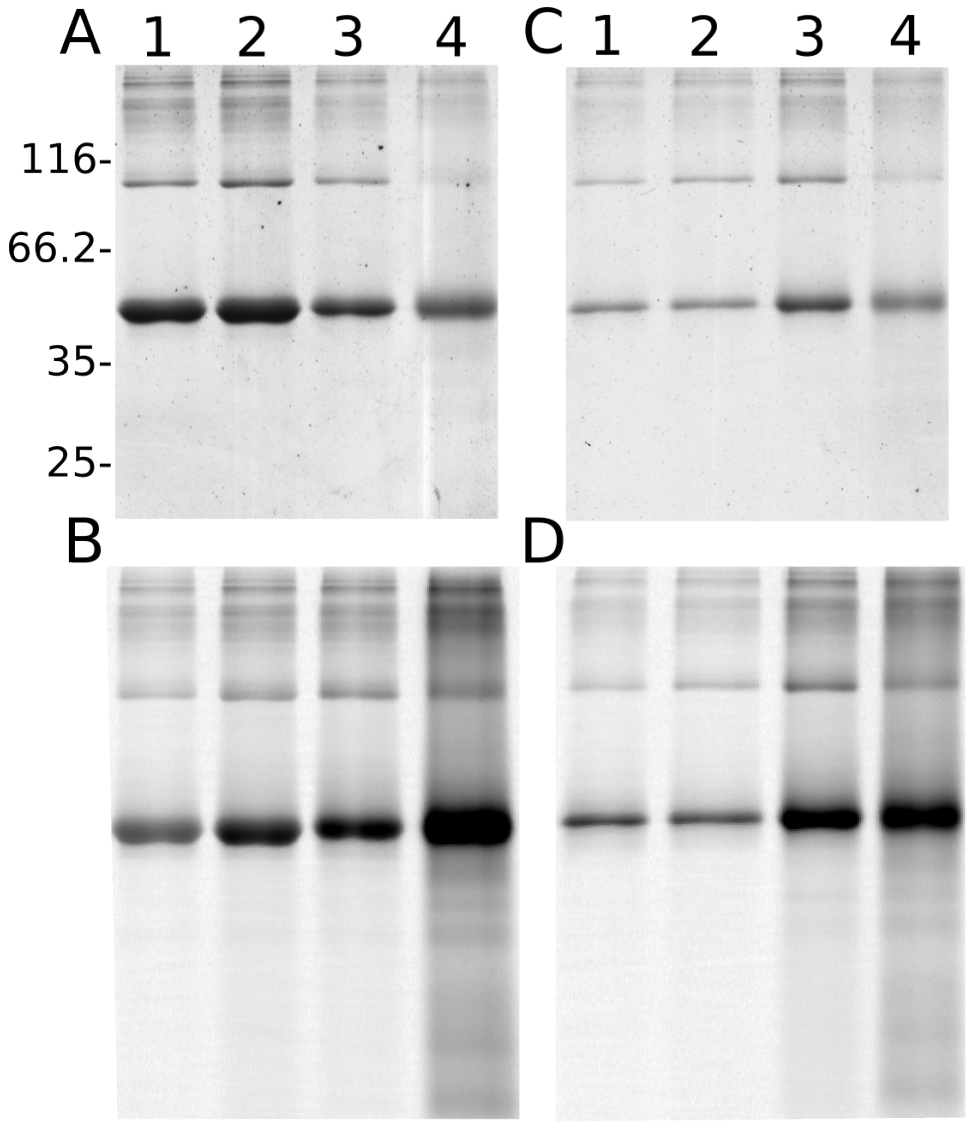

Figure 4.1: Representative gel images from 1DE of MCO treated BSA. Coomassie stain (A) and fluorecence image (B) of BSA after oxidation. Coomassie stain (C) and fluorescence image (D) of oxidised BSA after purification of carbonylated proteins by SPH. Pairs of images (AB and CD) were taken for the same gel. Lane numbers refer to sample number, 1 being the least oxidised and 4 the most oxidised.

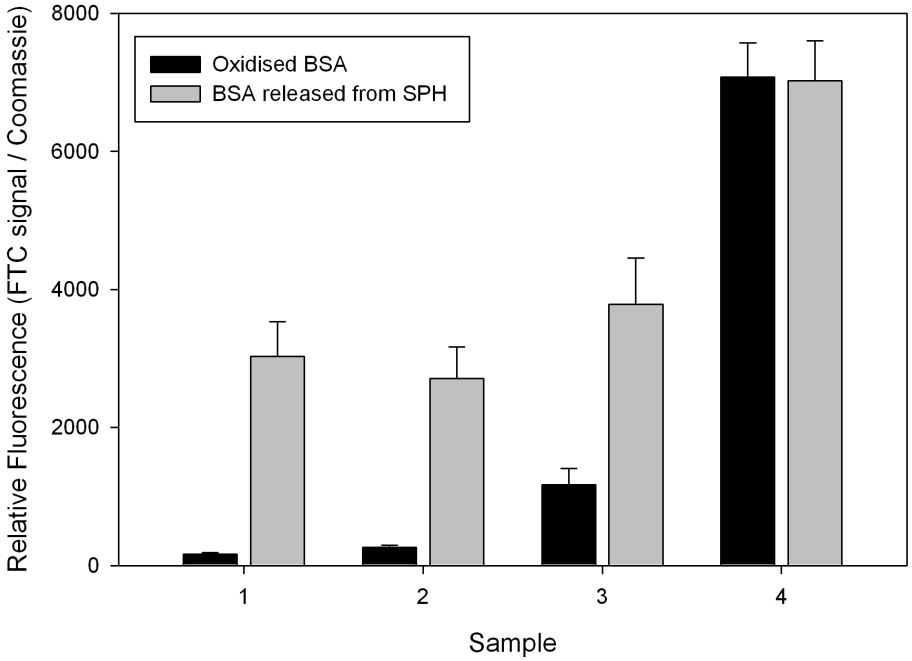

Figure 4.2: Relative carbonyl content of BSA prior to and after SPH isolation. Ratio is the fluorescence intensity of FTC labelled BSA divided by the coomassie intensity

4.3.2 Carbonylated protein isolation

In 1DE, the proteins released from SPH beads present a profile similar to that of the oxidised BSA (Fig. 4.1, C and D). There is an increase of the relative importance of the 84 kDa band and a presence of the same lower mass bands in the most oxidised sample. Relative fluorescence of isolated BSA was in the range of 3000 arb. unit up to sample 3, after which it drastically increased to 7024 arb. unit, a value equivalent to the one observed before SPH purification for this sample (Fig. 4.2).

4.4 Discussion and Conclusion

4.4.1 Metal catalysed oxidation

Prior to purification by SPH, BSA was oxidised by MCO. In the present protocol, ascorbic acid reduces Fe(III) and molecular oxygen to Fe(II) and peroxide, which then yields hydroxyl and superoxide radicals, two potent reactive oxygen species (Stadtman, 1991). This MCO reaction has often been used to carbonylate proteins in order to understand the mechanism and specificity of amino acid oxidation, particularly by the mixed-function oxidation system (Stadtman, 1991; Maisonneuve et al., 2009). The level of oxidation was controlled by varying the quantity of ascorbic acid and $FeCl_3$ and carbonylation was measured by labelling with FTC and subsequent 1DE. There was a clear increase in the relative carbonyl content of BSA as oxidation progressed (Fig. 4.2). The increase in carbonyl content was not linear, most likely because carbonylation altered the secondary structure of the protein, facilitating further carbonylation (Maisonneuve et al., 2009).

4.4.2 Carbonylated protein isolation

In order to study the redox-proteome, there is a strong interest in developping direct purification methods for carbonylated proteins in order to enrich those biologically significant targets of oxidative stress (Fedorova et al., 2013). To this end, we used a SPH based method to purify whole carbonylated proteins. The present results show that BSA oxidised to different levels by MCO could be enriched in carbonyl content. Interestingly, the relative carbonyl content of the purified proteins was the same for all samples except the most oxidised (Fig. 4.2). This indicates that BSA oxidises up to a certain level before carbonylation renders more sites available, leading to another level of oxidation. This is confirmed by the fact that the relative carbonyl content of sample 4 was the same before and

after SPH purification, indicating that it was not possible to enrich the sample in carbonyls further, most likely because all proteins were carbonylated to a similar extent. These results are only from the analysis of whole BSA, and do not take into account fragments or multiplexes of the protein. Fragments and multiplexes are present, especially in the most oxidised sample, but do not seem to form a major portion of the sample when considering their relative intensity in the 1DE profile (Fig. 4.1).

In conclusion, this study demonstrates the first application of SPH to purify whole proteins containing primary carbonyls generated by MCO. While SPH has been used on peptides previously (Roe et al., 2010; Rauniyar and Prokai, 2011; Han et al., 2012), it was always to study secondary carbonyls, HNE modified samples or with a specific interest for oxylipid protein conjugates. The present approach yields MCO carbonylated proteins in an unlabelled state, enabling direct analysis by mass spectrometry or future labelling prior to gel based separations, as was demonstrated here with FTC and 1DE. The use of covalent chromatography also enables washing the proteins in denaturing conditions, thus removing any interference from non-oxidised proteins bound to carbonylated proteins, as is the case with immunoaffinity or biotin-avidin based systems. However this method was only tested on simple, chemically oxidised, samples and tests with more complex mixtures of biological origin are in order to demonstrate its validity.

Chapter 5

Discussion

5.1 Measurement of biochemical parameters

The bulk of this thesis is concerned with the study of the toxicity of prooxidant compounds to the cladoceran *D. magna*. To this effect, toxicity of $CuCl_2$, paraquat, $AgNO_3$ and AgNP was first measured at the organism level using a standard immobilisation assay (OECD, 2004). As this was run as a preparatory step for further experiments, the results (p.27, 44) were not unexpected, except for the noteworthy fact that AgNP are less toxic than silver ions in the standard conditions used. The measured copper toxicity is lower than expected, which is due to the presence of EDTA in the medium, a known issue with chelator-containing media (Guilhermino et al., 1997).

Following the immobilisation assays, three concentrations were chosen for each compound (four for AgNP), going from one tenth to half the measured EC50. This was done in order to put the organisms under significant oxidative stress, while avoiding excessive mortality. Some mortality was still observed for exposures at the highest concentration of all compounds, but immobile organisms were not sampled for biochemical measurements. Mortality was under 10 % except for two of the $AgNO_3$ and one of the AgNP exposures, where it reached about 30 %.

In order to link the proteomics results to standard biochemical measures of oxidative stress and oxidative lesions, the activity of the enzymes catalase, GST and GAPDH was measured. These enzymes are often associated with oxidative stress. This was combined with the measurement of the carbonyl and thiol content of proteins using fluorescent dyes and 1DE. Although not directly suitable for absolute measurements, gel-based fluorescent detection of protein thiols (Baty et al., 2002) and carbonyls (Chaudhuri et al., 2006) is a good way to compare relative modification content and it is possible to achieve quantitative measurement for purified proteins, thus indicating that only an appropriate (however complex) control is required to enable quantification.

The measurement of the enzymatic activities only yielded one significant result: a reduction of catalase activity at low and intermediate paraquat concentrations (Fig. 2.1). Neither of the measured enzyme was thus affected by copper, AgNP and silver ions. Although unexpected, this result shows how using antioxidant systems to detect OS can be problematic. Indeed, antioxidant defenses can be overwhelmed in conditions of oxidative stress and show very different patterns of response over time, making the interpretation of the measure of a few antioxidant systems difficult (Regoli et al., 2002). To circumvent this problem and obtain a better picture of the redox status of a cell, measures of oxidative lesions – which are "metabolic endpoints" of OS – enable the integration of the redox status by detecting the level at which cellular components are exposed to ROS. In the case of proteins, carbonylation is the main product of ROS exposure, and it presents the advantage of being mainly irreversible. Although this simplistic view presents many caveats and is challenged (Wong et al., 2013), the fact remains that measurement of lesions through which cells detect oxidative stress is of high physiological significance to the organism, being of the level of the metabolome. Another protein modification which fulfills, although not as well, this requirement is oxidation of thiols. Thiols are readily oxidised by vari-

ous oxidants (Eaton, 2006) and act both as sensors and buffers of ROS in cells. Changes in the protein thiol content can thus be seen as a measure of the level of oxidative stress, although the "picture" is a more instantaneous one than with carbonyls, as the reversibility of most thiol oxidations means that changes in their redox state integrate a shorter time-scale than those of protein carbonyls.

The measured PTM did yield more significant results than the enzymatic activities. In samples exposed to AgNP, thiol and carbonyl content were both significantly increased (Fig. 3.1). This is surprising as oxidative stress normally leads to a decrease in protein thiols, and the increase in carbonylation is a clear sign of ROS damage. As protein thiols also increased after treatment with $AgNO_3$, the increase of protein thiols observed in both treatments is likely a response to the silver exposure, and potentially a sign of increased antioxidant defenses (which will tend to maintain thiols in a reduced state). Yet, the significant increase of the carbonyl content after AgNP exposure indicates an increased production of ROS, which was sufficient to overwhelm the cellular defences and cause protein oxidation. It would be expected in such a scenario that thiols would also be oxidised, as they are sensitive to the same ROS that generate carbonyls, but the oxidation of thiols is reversible, it is thus possible that thiols were indeed oxidised, but not to the point of overloading the repair machinery.

Generation of oxidative stress is a widely accepted mechanism of toxicity for nanoparticles of all types, and finding more signs of OS for AgNP rather than silver ions is not surprising, silver acting mostly through other means (Bianchini and Wood, 2003). It is much more surprising to find no sign of change for the measured enzymes or protein PTM following the exposure to copper and paraquat, two known prooxidants actually used in this study as model prooxidants. This lack of response, while the same enzymes have been shown to respond to those compounds in *D. magna* cannot be explained by the present experiments. Potential explanations may be linked to a different bubbling regime between laborato-

ries, as bubbling affects oxygen levels in the tanks, potentially affecting oxidative stress. It could also be a result of different resistances between the daphnia strains.

In the exposition to copper and paraquat, no sign was observed in the measured PTM, indicating that no major OS was present. The slight reduction of catalase activity by paraquat may be a result of oxidative damage (Mayo et al., 2003), but it could not be observed at the protein level nor in the subsequent proteomic study. More measurements, with a focus on time and dose-response would be required to obtain a clearer picture of the induction of OS by those two compounds. They were found to be prooxidants in *D. magna* in a previous study, though younger organisms were used and the exposure was longer (Barata et al., 2005b).

As the above biochemical assays were performed to compare the proteomic results to more standard biomarker measurements, this portion of the experiment was not as fruitful as hoped. It does help to highlight the complexity of biochemical responses, even to simple one-compound exposures. Yet, they do shed a light on the proteomic results below in indicating that *a priori* molecular markers may sometimes lack the sensitivity to demonstrate the presence of a response, while hypothesis-free approaches can demonstrate a specific response, even if it may be difficult to link it with the mechanism of action of the toxicant with the current knowledge.

5.2 Gel-based redox-proteomics

In order to better compare results between the experiments and enable easier linking of features with proteins identified in different experiments, all gel images obtained with one stain were aligned to a single reference gel image, which was chosen for practical and image quality reasons within the controls of the AgNP

exposure. The same gel was used to align the coomassie and fluorescence image, thus making it easier to link results from both stains after analysis. It is thus possible to confirm that very few features were common between the experiments. It is of note that, for the sake of publication, features were renumbered separately for chapters 2 and 3. For the present discussion, feature numbers will be kept as for the above chapters, with a prefix added to the feature number. Thus features of interest from Chapter 2 are marked 2.fxx and those from Chapter 3 as 3.fxx. All features of interest are listed in Table 5.1, not just those which were identified by MS but all those answering the significance criteria of a fold change > 1.5 and $p < 0.05$ in ANOVA.

Including all exposures, there were 32 cases of changing protein volumes (16 decreases and 17 increases), 24 cases of FTC fluorescence volume changes (16 decreases, 6 increases and one showing a decrease followed by an increase), including seven cases where both protein and fluorescence volumes changed. Forty nine features thus presented change when all the treatments are considered, and only four features changed following exposure to more than one of the compounds tested, showing the specificity of the observed responses and leaving 45 unique features presenting change in at least one treatment.

The use of a redox-proteomics approach, in this case labelling with FTC, has enabled the detection of 17 more changes of interest, an increase of almost 40 % from the results obtained by coomassie. Considering the usual difficulty to identify proteins from environmentally relevant species, the possibility to increase by such an extent the number of detected changes is not to be overlooked. Especially since this approach fits easily within a normal gel-based proteomics workflow, with the main hurdle being the availability of a fluorescence scanner. The actual labelling and washing of unbound labels requries about two hours more of waiting time before rehydration of the Immobilised pH gradient (IPG) strips.

Table 5.1: Features of interest found in two-dimensional electrophoresis[a]

Protein[b] (feature[c])	**$CuCl_2$**	**Paraquat**	**$AgNO_3$**	**AgNP**
Vitellogenin-like protein (3.f1)	– / –	– / –	– /-1.7	– / –
Unidentified (3.f2)	– / –	– / –	– / –	1.9 / –
Unidentified (2.f1)	– /-1.8	– / –	– / –	– / –
Neural Endopeptidase 24.11 (2.f2)	-2.0/-2.5	– / –	– / –	– / –
Vitellogenin fused with superoxide dismutase (2.f3, 3.f3)	– / 2.6	– / –	1.8 / –	– / –
Vitellogenin fused with superoxide dismutase (2.f4, 3.f4)	– / 2.5	– / –	– / –	1.5 / –
Vitellogenin fused with superoxide dismutase (3.f5)	– / –	– / –	– / –	-1.6/-1.7
Unidentified (3.f6)	– / –	– / –	– / –	– /-2.6
Hemoglobin (3.f7)	– / –	– / –	– / –	1.6 / –
Unidentified (3.f8)	– / –	– / –	– / 2.0	– / –
Unidentified (2.f5, 3.f9)	2.3 / 2.0	– / –	– / –	2.0 / 1.7
Vitellogenin-like protein (2.f6)	2.1 / 1.6	– / –	– / –	– / –
Vitellogenin-like protein (3.f10)[d]	– / –	– / –	– / 1.4	– / –
HSC71 (2.f7)	-2.1/ –	– / –	– / –	– / –
Unidentified (3.f12)	– / –	– / –	-1.8/ –	– / –
Unidentified (3.f13)	– / –	– / –	– / –	2.2 / –
Unidentified (3.f14)	– / –	– / –	-1.6/ –	– / –
Sb:cb283 protein, partial (2.f8)	-1.8/-1.6	– / –	– / –	– / –
Unidentified (2.f9)	-1.7/ –	– / –	– / –	– / –
Heat shock protein 70 (2.f10)	– /-1.7	– / –	– / –	– / –
Unidentified (2.f11)	-1.5/ –	– / –	– / –	– / –
Unidentified (2.f12)	-1.6/ –	– / –	– / –	– / –
Unidentified (2.f13)	-1.5/ –	– / –	– / –	– / –
Unidentified (2.f14)	-1.5/ –	– / –	– / –	– / –
Heat shock protein 70kDa, partial (2.f15)	-3.0/ –	– / –	– / –	– / –
GG12661 (2.f16)	-2.6/ –	– / –	– / –	– / –
Unidentified (2.f17)	1.8 / –	– / –	– / –	– / –
Unidentified (2.f18)	1.9 / –	– / –	– / –	– / –
Unidentified (2.f19)	-1.5/ –	– / –	– / –	– / –
Unidentified (2.f20)	2.8 / –	– / –	– / –	– / –
Vitellogenin fused with superoxide dismutase (3.f15)	– / –	– / –	– / –	– /-1.8
Vitellogenin fused with superoxide dismutase (2.f21)	1.6 / –	– / –	– / –	– / –
2-phospho-D-glycerate hydrolase (2.f22)	– / –	– /-1.7	– / –	– / –
Actin related protein 1 (2.f23)	1.4 / –	– / –	– / –	– / –
Unidentified (3.f16)	– / –	– / –	– / –	– /-2.0
Fructose 1,6-bisphosphate aldolase (2.f24)	– / –	1.4 / –	– / –	– / –
Arginine Kinase (2.f25)	– /-1.7	– / –	– / –	– / –
Unidentified (2.f26, 3.f17)	2.0 / –	– / –	– / –	1.6 / –
Unidentified (2.f27)	– /-2.3	– / –	– / –	– / –
Trypsin 208, partial (2.f28)	-1.8/-1.9	– / –	– / –	– / –
Translational elongation factor-2 (2.f29)	1.5 / –	– / –	– / –	– / –
14-3-3 protein epsilon (3.f18)	– / –	– / –	– /-2.0	– / –
fk506-binding protein (2.f30)	– /-2.0	– / –	– / –	– / –
Actin (2.f31)	-1.9/ –	– / –	– / –	– / –
Chymotrypsin-like protein (2.f32)	– /-2.0	– / –	– / –	– / –

[a] Highest significant fold change relative to control, values are Coomassie/Fluorescence

[b] The proteins are presented in the same order as the numbering withing the Samespot software, which reflects position on the gel (numbers increase from the top left corner to the bottom right corner, following horiztonal lines)

[c] The feature numbers refer to previous chapters and are of the form : Chapter.fxx

[d] The change for this feature was in both directions, a decrease at low concentration and an increase at high concentration

In addition to the discovery of 17 new changes, labelling with FTC allowed us to confirm with a second dye seven of the changes observed with colloidal coomassie staining. Indeed, seven features showed significant changes at the protein and carbonyl level, and in every case the changes were parallel and of similar intensity, indicating that both dyes measured the same change, that of protein quantity, and that the protein maintained a similar level of carbonylation. For the other 38 detected changes, only protein or carbonylation changed. Although it is not possible to conclude for every feature that proteins and carbonylation level changed independently from one another (due to technical limitations), it is more than likely that many features presented changes at the carbonyl level without changing at the protein level, and *vice-versa*. A more complete picture could be reached if fluorescence levels were normalised by protein stain levels for each features, but this is technically complex due to the different sensitivity and linear ranges of both dyes, along with the fact that carbonylation is not uniform between different proteins. The best way to obtain such a picture is likely through mass spectrometry, and there is no standard method available at the moment to detect carbonyls and determine carbonylation levels (Fedorova et al., 2013), especially since quantification of carbonyls requires previous quantification of proteins, something not yet easily achieved in proteomics.

5.2.1 Protein identifications

Gel plugs from the 45 features discussed above were sent for identification by MALDI-TOF/TOF at the Instituto de Technologica Química e Biológica (Universidade Nova de Lisboa, Portugal). Twenty-five (56 %) features could be identified (Tables 2.1 and 3.2), of which seven idenfications were redundant (2.f3, 2.f4, 3.f3, 3.f4 and 3.f5 were the same protein, as well as 2.f6 and 3.f10), giving a total of 20 unique identifications. Six of the identified proteins were from *D. magna* sequences and 12 were identified as hypothetical proteins from the *D.*

pulex genome. The other four proteins were identified as coming from arthropods (3) or fish (1, from *Danio rerio*). As no function was directly known for the *D. pulex* hypothetical proteins as well as for feature 2.f7 (an uncharacterized protein), bioinformatics tools were used to infer putative biological functions for the proteins. All sequences were studied for similarity with known proteins using blastp (Altschul et al., 1997) and DELTA-BLAST (Boratyn et al., 2012) as well as for the presence of conserved sites and domains and sequence similarity to protein families with DELTA-BLAST and the Interproscan tool (Quevillon et al., 2005). Although annotations are available for the *D. pulex* hypothetical proteins, this approach was chosen to remain consistent with the analysis of the uncharacterised protein (2.f7) which also required a more in depth functional study. Annotations of the hypothetical proteins are available from the JGI Genome Portal (http://www.Jgi.doe.gov/Daphnia/) for the *D. pulex* gene annotations. The approach followed here yielded similar results to the annotations found on the JGI website. Results of the bioinformatics analysis are presented in Tables 2.2 and 3.3 and the protein names presented in Table 5.1 stem from the MS identification and following bioinformatics analysis.

A first glance at Table 5.1 reveals that some features that are nearby on the gel contain the same protien. Indeed, all the redundant protein identifications listed above are found within two protein trains. Interestingly, as discussed in Chapter 3, all these features were identified as vitellogenins. These trains are likely formed of various vitellogenin maturation products, the presence of "vitellogenin trains" is expected as vitellogenin is known to be heavily modified during its maturation, notably through cleavage and multiple phosphorylation (Kato et al., 2004), which will affect its molecular weight and pI. Also, the direction of change and fold changes of vitellogenin-containing features within a train did not behave uniformly, with one feature being found to be significantly altered by one treatment and not the other (*e.g.* 3.f3-5). In addition to the two vitellogenin trains,

features 2.f21 and 3.f15 are neighbours and both contain a vitellogenin fused with SOD (Tables 2.1 and 3.2), although the proteins were identified as two different vitellogenin-SOD. It is not possible to determine wether they are actually the same or different proteins, as *DmagVTG1* and *DmagVTG2* (the two genes the proteins were identified as) present a high sequence similarity (Tokishita et al., 2006), and the low sequence converage (14 and 15 %) is not sufficient to discriminate between the two proteins. Thus, not all the vitellogenin identifications are redundant, and there is a high presence of vitellogenins within the identified proteins, with eight features spread over most of the gel, and representing ten of the 49 observed feature changes (20 %).

This corroborates the idea presented above (page 45). Indeed, the PES of vitellogenins appears from the present results to be specific to the stressor. There would thus be an interest in studying further the applicability of the vitellogenin proteome as a stress biomarker. Although already considered by Jubeaux et al. (2012), there is a clear advantage in working with a gel-based approach as much more information can be gleaned from the maturation process of various vitellogenins rather than only their expression level. In this matter the present results are in agreement with the conclusions of Gündel et al. (2007) that present lipovitelin derivatives as sensitive stress indicators in zebra fish enbryos.

When looking at all the exposures together, vitellogenin is the only functional category of proteins to be significantly affected by more than one compound. Even though the PES of vitellogenins is unique to each compound, it demonstrates that the sensitivity of vitellogenin responses is in no way specific to a compound, in agreement with the conclusions of Hannas et al. (2011), who showed that changes in vitellogenin gene expression are not linked to a specific category of toxicants. Although this is a statement against the specificity of changes in vitellogenin maturation, this also means that the other functional categories observed were observed only for a single tested compound, indicating a measure of specificity in

the present exposure regimen.

Thus, hemoglobin protein levels were only modified (increased) by the exposure to AgNP. This is also the only compound for which a clear sign of OS was present, *i.e.*: increased carbonylation levels as measured by 1DE. There may be a mechanistic link between those two observations as hemoglobin expression is under control of the hypoxia-inducible factor 1 and ROS can induce its activity (Becker et al., 2011).

Following $AgNO_3$ exposures, the carbonylation level of a 14-3-3 protein family member was reduced. This was the only response specific to the treatment (all other modified features containing vitellogenins) and it is difficult to link it to the mechanism of action of silver ions as 14-3-3 proteins are regulatory proteins with very diverse functions (van Hemert et al., 2001). One possible, highly speculative, functional link between changes in 14-3-3 proteins and silver toxicity may be derived from the fact that, in a euryhaline crab, the sodium pump (Na^+/K^+-ATPase) α-subunit presents a variant containing a 14-3-3 binding site, which is likely involved in the transfer of the sodium pump from the endoplasmic reticulum to the cell membrane thus activating the sodium pump (Jayasundara et al., 2007). As the main mechanism associated with silver toxicity is known to be disruption of osmoregulation (Bianchini and Wood, 2003), the change in the 14-3-3 protein may be linked to the toxic effects of silver or to an adaptative mechanism from the daphnid. This is, of course, highly speculative as no functional information is available on the 14-3-3 proteins and its potential link to osmoregulation in daphnids.

Only two features were significantly modified by the paraquat exposures, and, luckily, both were identified. In this case, none of them contained a vitellogenin, indicating that vitellogenin was not responsive to paraquat exposures. The two responsive proteins (2-phospho-D-glycerate hydrolase – 2.f22 – and fructose 1,6-bisphosphate aldolase – 2.f24) are involved in glycolysis, a result in accordance

with the known mechanism of toxicity of paraquat, as it impedes cellular respiration by accepting electrons from the electron transport chain (Vicente et al., 2001), thus reducing mitochondrial efficiency and leading to ROS production. The activity of both enzymes was likely increased by exposure to paraquat (enolase protein levels increased and aldolase carbonylation decreased), those activities would warrant measuring directly for a more in depth study of paraquat in daphnids. As discussed earlier (p. 39) it is likely that the changes in glycolytic enzymes represent a sign of higher ATP requirements, and thus an increase in glycolysis. The apparent lack of oxidative stress following paraquat exposure and absence of changes of the measured enzymes (especially catalase) is in contradiction with the results of Barata et al. (2005b). The discrepancy between the two studies cannot be easily explained with the present results as, at the organism level (EC50), the observed toxicities were similar. It is probable though that the differences in biochemical measurements stem from the different exposure times (24h *vs* 48h) and age of the exposed daphnids (7 days old *vs* neonates).

Although the results obtained here for the copper exposures disagree significantly from those of Barata et al. (2005b) – *i.e.*: lack of response of the measured enzymes and absence of oxidative stress – the proteomic study yielded much more diverse and interesting results for this compound than for the other three tested. Indeed, on top of vitellogenins (of which four features were affected, always with an increase in protein and/or carbonylation), three other functional protein categories were significantly affected : Proteolytic enzymes, chaperones and actin filament proteins.

Four chaperones saw their protein expression level (2.f7, 2.f15) or carbonylation (2.f10, 2.f30) reduced by copper exposures. Of these, three are HSP70 family members, and one is a cyclophilin (peptidyl-prolyl cis-trans isomerase). It is surprising to see the expression of two HSP70s being reduced under metal stress, as this family of proteins is linked to the heat-shock response (HSR), which is

normally induced by metal stress (Feder and Hofmann, 1999). Yet, it has been observed in daphnids that a reduced HSR is associated with increased resistance to cadmium (Haap and Köhler, 2009). As cyclophilins are also associated with the HSR (Mathew and Morimoto, 1998), the detection of a less carbonylated cyclophilin may be linked to the response of HSP70, potentially indicating higher turnover of the protein. A second functional category linked to the HSR is also strongly represented in this study. Indeed, four proteolytic enzymes were affected by copper, all of them presenting a decrease in protein level and/or carbonyl content. Two are well known digestive enzymes in daphnids (trypsin 2.f28 and chymotrypsin 2.f32), one is a member of the peptidase M13 family (2.f2) and the last is a leucine aminopeptidase family member (2.f8), both enzymes whose function in daphnids is not known, but is likely more related to intracellular protein metabolism than to digestion. Proteases are usually upregulated under the HSR (Mathew and Morimoto, 1998), and three of the proteases found in this study present reduced protein levels (as well as carbonyl levels) while one shows only a reduction of carbonylation.

Taken together, the response of the chaperones and proteolytic enzymes indicate that the HSR is likely inhibited by the copper exposures. Although no report of such an inhibition could be found in the literature following metal exposure, it may be linked to the fact that lower basal levels and lower induction of the HSR have been associated with increased resistance to metals (Haap and Köhler, 2009) though the mechanistic link between reduced HSR and increased resistance is not known, and counterintuitive.

The other functional group of proteins found to be modified was that of actin-filament related proteins. Indeed, two actins (2.f23 and 2.f31) and one alpha-actinin (2.f16) saw their protein levels affected by the copper exposure. Actin filaments are a fundamental component of the cytoskeleton and, as such, are involved in cell structure, motility, division and in cellular trafficking. Alpha-

actinin, showing reduced expression following exposure, cross-links actin filaments together or with membrane-bound proteins (Djinović-Carugo et al., 1999). Interestingly, one of the actin proteins found here (2.f23) is downregulated while the other (2.f32) is upregulated by the exposures, likely indicating a change in the types of actin expressed, a possible adaptative mechanism to the presence of high copper concentrations. The specific isoform of the detected actins cannot be inferred from the results obtained here, the sequence of daphnid actins being too similar (Schwerin et al., 2009). Lack of information on daphnid actins and more precise identifications prevents giving a mechanistic explanation for their behaviour, although they are known to be affected by cold acclimatation (Schwerin et al., 2009) and predator-stress (Pijanowska and Kloc, 2004; Schwarzenberger et al., 2009).

Aside from the above-described groups of proteins, arginine kinase (2.f25) and TEF-2 (2.f29) were also affected by copper. Arginine kinase is the main enzyme of the only phosphagen system of arthropods (Ellington, 2001) and is thus an important part of the energy metabolism of the organism. The lowered carbonylation level may indicate an increase of its activity or a higher turnover rate of this enzyme. As most of the features showing a significant change in carbonylation levels showed a reduction following copper exposures (nine out of 13), it is possible that overall protein turnover was increased by the exposure. This may be linked to increased expression of TEF-2 observed. As protein carbonylation is generally irreversible, the only way that carbonylation levels can be reduced is through degradation of oxidised protein and synthesis of new proteins to replace them. If turnover is indeed increased (as the carbonyl reduction is not associated with a protein level reduction), this could lead to changes in the expression of the translation machinery, of which TEF-2 is a part. Important changes in the expression of mRNA involved in protein synthesis has already been observed for daphnids exposed to cadmium (Connon et al., 2008), although

they observed a reduction for TEF-2 at high concentrations while here a slight reduction is observed at the lowest concentration followed by an increase with higher concentrations (Table 2.3).

Taken together, the observed response for the copper exposure does not present signs of OS, nor insight into the mechanism by which it exerted toxicity on the daphnids, but presents a potentially interesting (adaptative?) response to the stress by a reduction in proteins from the heat-shock response, a counter-intuitive response warranting further studies as it does seem selected in arthropods living in metal-contaminated environments (Haap and Köhler, 2009).

5.2.2 Conclusion of the proteomics studies

The results from the biochemical measurements and proteomic studies clearly differentiate between the four compounds tested, indicating that the PES is indeed specific to the toxicant. This is true even if only vitellogenins are considered, even though they are widely represented in the response of three of the compounds ($AgNO_3$, AgNP and $CuCl_2$) both as a functional group and at the level of some features. For all other proteins identified, each responded only to one stressor. The validity of PES has often been demonstrated (Bradley et al., 1994; Kimmel and Bradley, 2001; Silvestre et al., 2006), even in field studies (Vioque-Fernández et al., 2009) and it is one of the most promising applications of proteomics in ecotoxicology. The specificity of the responses observed in this study confirms this evaluation.

Identification of the differentially expressed proteins, as a subsequent step to PES determination, has not brought much insight into the toxicity mechanism of the tested compounds. Indeed, very little can be told from the present results about the toxicity mechanisms of AgNP and $AgNO_3$, and only general reactions of the organisms to copper and paraquat toxicity were observed. Although the understanding of the biochemical responses of daphnids in answer to chemical

stressors is of interest and important to elucidate, the often presented promise of biomarker discovery from omics cannot be fulfilled by the present study. Indeed, although results such as those for copper above open the door to more in depth studies of toxicity which may lead to finding a sensitive and specific protein to detect the presence of the compound, this requires much more efforts than was deployed here, or in most omics studies.

This issue is actually wider than proteomics, as "the extent to which biomarkers are able to provide unambiguous and ecologically relevant indicators of exposure to or effects of toxicants remains highly controversial" (Forbes et al., 2006). There are thus still many questions, and doubts, concerning the usefulness of biomarkers in ecological risk assessment. Although approaches based on PES (*e.g.*: Vioque-Fernández et al., 2009), and machine learning (Bradley, 2012) could lead to a gain in sensitivity and specificity, this gain would have to be impressive to balance the increased cost and complexity of a proteomics-based environmental monitoring program. The suggestion of Jemec et al. (2010) to redefine the role of biomarkers to hazard identification rather than exposure assessment or risk management thus seems appropriate. Forbes et al. (2006) highlight that in order to better interpret and validate biochemical measurements, and link them to population level-responses, mechanistic links between the levels of organisation must be known. This is in line with the suggestion of van Straalen (2003) to shift towards stress ecology in the future of ecotoxicological research. Increased mechanistic understanding is also clearly within the scope of omics approaches, and although not giving rise to clear understanding of toxicity pathways, some of the results presented here (especially for copper), could give rise to more in depth studies to better understand the response of daphnids to toxicants.

The results presented in Chapter 3 demonstrate a place for proteomics in hazard identification. Indeed, the present study demonstrates that even though the immobilisation test would have discarded AgNP as less toxic than silver

ions, their toxicity follows a different mode of action, and more studies are thus required to evaluate potential interactions and environmental impacts of AgNP. It also highlights the fact that a simple measurement of silver concentration within an organism or environmental sample may miss the contribution of AgNP to contamination and toxicity. This is also confirmed by the transcriptomics study of Poynton et al. (2012), who demonstrate different modes of action for silver ions and AgNP, as well as an impact of the NP coating on gene responses of *D. magna*.

5.3 SPH enrichment of carbonylated proteins

The results presented in Chapter 4 demonstrate the potential of SPH to isolate whole proteins oxidised by MCO. Although the method requires further testing with more biologically-significant samples, there is promise in the use of a batch method to purify carbonylated proteins. Indeed, the low abundance of any single carbonylated species in the proteome means that their identification is difficult, especially against the background of more abundant non-carbonylated proteins. In line with results obtained in our laboratory with the thiol proteome (Hu et al., 2010), much can be hoped for the carbonyl proteome. The possibility of trapping carbonylated proteins directly from a homogenate and subsequent release of unlabelled carbonyls is likely to facilitate gel-based studies or direct MS detection and characterisation. The use of carbonyl tags could also be considered in order to facilitate release of the proteins along with subsequent analysis.

Beyond the possibility of its application to complex samples, many questions are still left considering this protocol. Indeed, as it is based on a chemical equilibrium, it is pertinent to wonder how much of the carbonylated proteins are released from the resin, and how this could be affected by using derivatisation agents to favour release. Also, the specificity and efficiency of binding of different

carbonyl moieties are worth characterising, as is the case for derivatisation agents (Bollineni et al., 2013).

Chapter 6

Conclusion

The technologies and industries that make modern society possible are altering the biosphere in many ways. Chemically this is done through the mobilisation of metals by mining and production of novel compounds. Ecotoxicology attempts to measure and predict the impact of the increased concentrations of xenobiotics on ecosystems, in the hope of mitigating the impact of xenobiotics on ecosystems and minimising future effects. In order to do so, a mechanistic understanding of the impact of pollutants is required and biochemical tools have been increasingly used to decipher the cellular effects of pollutants on ecologically-relevant organisms.

Following advances in genomics, proteomics were developed, enabling the study of the protein complement of cells. This opened the door to hypothesis-free approaches to hazard identification. As the methods were refined, the interest in subproteomes grew in order to obtain more specific responses and circumvent technical limitations. In ecotoxicology, the redox proteome is of particular interest. Not only are changes in the redox balance of cells often observed following exposure to toxicants, they are also associated with many natural environmental conditions (Lushchak, 2011). Redox-proteomics can thus help understand the interaction between environmental factors and anthropogenic perturbations.

Among ecologically relevant organisms, daphnids stand out because of the

amount of literature on their ecology, evolution, behaviour and, increasingly, subcellular responses. Indeed, the recent sequencing of the *D. pulex* genome (Colbourne et al., 2011) demonstrates the interest in daphnid biochemistry and opens the door to the application of omics tools to study members of this taxa.

This thesis expands on this interest by presenting the first application of redox-proteomics to study a daphnid (*Daphnia magna*). Fluorescent labelling of protein carbonyls prior to 2DE was used to quantify changes in the proteome following exposure to model prooxidants copper and paraquat. This demonstrated that both compounds affected daphnids differently, copper causing a reduction of the heat shock response and paraquat affecting the level and carbonylation of glycolytic enzymes.

Once applied to well known compounds, the approach was used to gain insight into the mechanism of toxicity of the emerging pollutant AgNP, in comparison with that of silver nitrate. We found a clear difference between the impacts of AgNP and silver ions on the daphnid proteome. This implies that both compounds follow different toxicity mechanisms, and thus that AgNP toxicity is not only a product of silver dissolution. There is cause for concern over the environmental release of AgNP, as environmental conditions and other pollutants will likely impact their toxicity differently than that of silver ions.

One element of particular interest is the fact that 20 % of the proteins identified in this project are vitellogenins. This indicates the high sensitivity of this oogenesis-linked protein, but also its lack of specificity to stressors. Yet, this means vitellogenins could be used to detect and differentiate stressors, as the PES of vitellogenins was unique to each of the materials studied. Another point of note is the reduction in the heat shock response following exposure to copper. As HSP70 are known to present lower basal levels in arthropods from metal-contaminated environments (Haap and Köhler, 2009), this appears to be more frequent than would be expected and warrants further study as an increased

resistance to metals following a decreased heat-shock response deserves studying.

This thesis demonstrates the usefulness of redox-proteomics to obtain more information from gel-based proteomics (increase of 40 % in the number of features of interest), even if no clear sign of oxidative stress was observed in the organism. Even though there can be doubts about the capacity of proteomics to lead to biomarker discovery, its usefulness in expanding our understanding of toxicity mechanisms and exploring the risk associated to novel contaminants is undeniable.

In order to expand on available methodologies to study protein carbonylation, we also demonstrate the possibility of using solid-phase hydrazide to enrich carbonylated proteins. BSA oxidised to various degrees by MCO could be purified by covalent chromatography and the underivatised carbonylated proteins could be released and studied by 1DE. The development of this approach could facilitate analysis of the carbonylated proteome and thus of the mechanisms of oxidative stress and redox homeostasis.

Appendix A

Collaborations

In the course of my PhD, I contributed to different projects centered on the use of gel-based redox-proteomics, mostly by sharing the methods used in the laboratory with collaborators. From this, two articles have been published to date. One is a review written by Prof. Sheehan and previous PhD students from the laboratory (Sheehan et al., 2012) to which I mainly contributed figures and tables. The other is the product of a collaboration with Dr. Wiebke Schmidt and Dr. Brian Quinn from the Galway-Mayo Institude of Technology (Schmidt et al., 2013). I took part in the 2DE experiments (though I did not perform most of them), data analysis and the writing process.

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FORUM REVIEW ARTICLE

Redox Proteomics in Study of Kidney-Associated Hypertension: New Insights to Old Diseases

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Abstract

Significance: The kidney helps to maintain low blood pressure in the human body, and impaired kidney function is a common attribute of aging that is often associated with high blood pressure (hypertension). Kidney-related pathologies are important contributors (either directly or indirectly) to overall human mortality. In comparison with other organs, kidney has an unusually wide range of oxidative status, ranging from the well-perfused cortex to near-anoxic medulla. ***Recent Advances:*** Oxidative stress has been implicated in many kidney pathologies, especially chronic kidney disease, and there is considerable research interest in oxidative stress biomarkers for earlier prediction of disease onset. Proteomics approaches have been taken to study of human kidney tissue, serum/plasma, urine, and animal models of hypertension. ***Critical Issues:*** Redox proteomics, in which oxidative post-translational modifications can be identified in protein targets of oxidative or nitrosative stress, has not been very extensively pursued in this set of pathologies. ***Future Directions:*** Proteomics studies of kidney and related tissues have relevance to chronic kidney disease, and redox proteomics, in particular, represents an under-exploited toolkit for identification of novel biomarkers in this commonly occurring pathology. *Antioxid. Redox Signal.* 17, 1560–1570.

Introduction

The kidney performs crucial physiological roles, including removal of nitrogenous waste materials (e.g., urea and uric acid), control of water, electrolyte, and acid-base balance in blood, and retention of useful components (e.g., glucose and amino acids). Plasma is filtered by kidneys (350–400 ml/100 g tissue per min), generating 150–180 liters/day ultrafiltrate, of which >99% is reabsorbed (69). Important renal pathologies include primary glomerular disease, diabetic nephropathy, renal allograft rejection, lupus nephritis, Fanconi syndrome, renal cell carcinoma, chronic kidney disease, and acute kidney injury. Definitive diagnosis of the underlying cause of disease can be difficult, so proteomics represent a promising novel diagnostic approach (53, 69). Chronic kidney disease affects some 11% of the general population and is evident when renal glomerular filtration rates are reduced (12, 17, 20). It is a significant risk factor in predisposition to cardiovascular illness and also in patients with pre-existing cardiovascular disease (20). Chronic kidney disease can progress to end-stage kidney disease, both of which are quantitatively important contributors to overall human mortality (72).

A key physiological consequence of kidney pathology is elevated blood pressure (hypertension) that can result in stroke and cardiac disease (20). Oxidative stress (Fig. 1) arises when levels of reactive oxygen or nitrogen species (ROS or NOS) exceed the cell/tissue's antioxidant capacity and has consistently been associated with hypertension (51, 60) and stage of renal malfunction (15). ROS/RNS can interact directly with important cell components such as lipids, nucleic acids, and proteins (82), and lead in particular to glomerular filtration barrier injury (52).

In comparison with other organs, the kidney has an unusually wide range of oxidative status, ranging from the well-perfused cortex to near-anoxic medulla (Fig. 2). The medulla and cortex also fulfill distinct functions. In the cortex, fluids and electrolytes are filtered from proteins at the glomerulus, and glucose, electrolytes, and water are reabsorbed along the nephron. The medulla, on the other hand, is almost anoxic and has the principal function of concentrating urine. Oxidative/nitrosative stress arising from ROS/RNS is thought to be differentially regulated in the cortex and medulla (55). NO-mediated signaling is more important in medulla, but cortex may be more liable to ROS-induced damage (60). Onset of kidney dysfunction seems to arise from progressive loss of redox homeostasis associated with overproduction of ROS/RNS, with consequent effects on renal proinflammatory/proapoptotic/profibrotic pathways. These lead to changes in vascularization, fibrosis, and diminished

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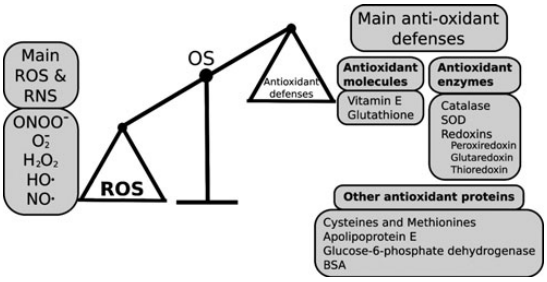


FIG. 1. Oxidative/nitrosative stress. Under conditions of redox homeostasis, antioxidant defenses exceed ROS/RNS concentrations. When this balance tilts, so that ROS/RNS exceed antioxidant defenses, a situation of oxidative/nitrosative stress ensues.

kidney function (51, 52, 60). Proteins are key quantitative targets for ROS/RNS; many proteins are known biomarkers of kidney pathology (17). This review focuses on redox-related proteomic studies of chronic kidney disease and related conditions. We will describe general approaches taken in human samples and associated model systems and assess the potential of redox proteomics to contribute new insights to chronic kidney disease.

Triggers for Oxidative/Nitrosative Stress in Kidney Disease

Physiological factors external to kidney are capable of triggering events within the organ, resulting in excess production of ROS/RNS and causing oxidative/nitrosative stress (Fig. 3). This can affect proteins, and oxidative stress increases with the stage of chronic kidney disease (15). Regardless of the initial cause, fibrosis is a common proximate cause for end-stage kidney disease, and proteomic profiling may provide novel early biomarkers with sufficient predictive power for clinical outcomes (32, 59). Hyperglycemia due to diabetes mellitus is commonly associated with onset of hypertension; this is the most common clinical cause of chronic kidney disease (12). Chronic kidney disease represents a persistently inflamed state often associated with pro-inflammatory stimuli such as interleukins-1, −6, −18, tumor necrosis factor alpha (TNF-alpha), and other cytokines (49). This inflammation contributes to progression of the disease by increased activity and production of adhesion molecules that contribute to adhesion of T cells and their migration into the interstitium, leading to attraction of profibrotic factors (78). Growth factors external to kidney contribute to the process of scarring or fibrosis which features excessive deposition of extracellular matrix causing loss of renal function (38). Fibrosis has been suggested to be a process of phenotypic transition from epithelium to mesenchyme (1), although the definitiveness of this has recently been questioned (34). The role of the endocrine or circulating renin–angiotensin–aldosterone system (RAAS) in stimulating fibrosis during chronic kidney disease is well established (52, 83). Decreased renal perfusion causes secretion of renin from the juxtaglomerular apparatus of the kidney. This cleaves circulating angiotensinogen from liver to form the ten-residue peptide hormone angiotensin I. This, in turn, is cleaved by removal of two C-terminal residues catalyzed by angiotensin-converting enzyme (ACE) from lung, to form angiotensin II. This binds to angiotensin receptors, triggering effects within the kidney, including reabsorption of sodium and protein. Angiotensin II also triggers release of aldosterone from adrenal glands and has increasingly been associated with numerous other proinflammatory functions (5). Overexpression of angiotensinogen attenuates apoptosis in model animals (21). Overstimulation of the RAAS cascade leads to kidney damage, resulting in diminished function (52, 83). All RAAS components are also produced locally in kidney, and it

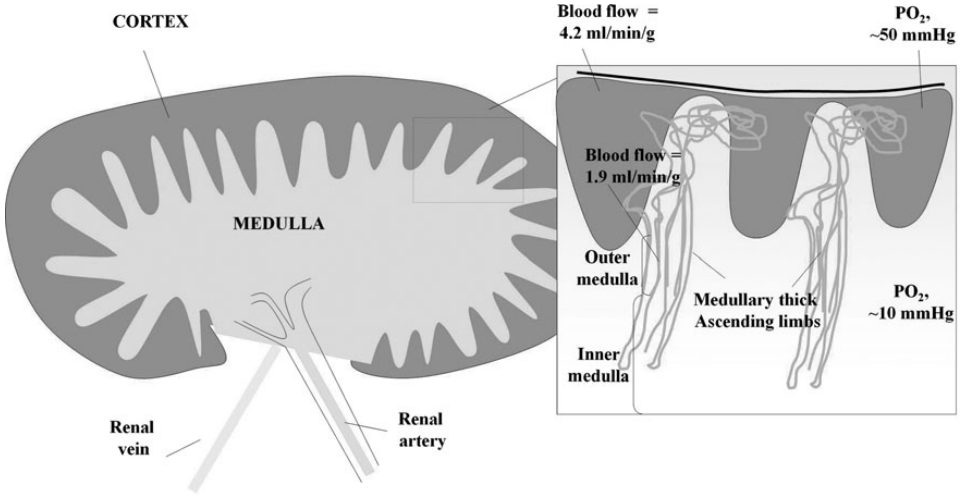


FIG. 2. Blood flow and oxygenation in the kidney. The cortex is well-perfused with high levels of oxygen. The medulla has a lower blood flow-rate with correspondingly lower oxygen levels (adapted from Ref. 76).

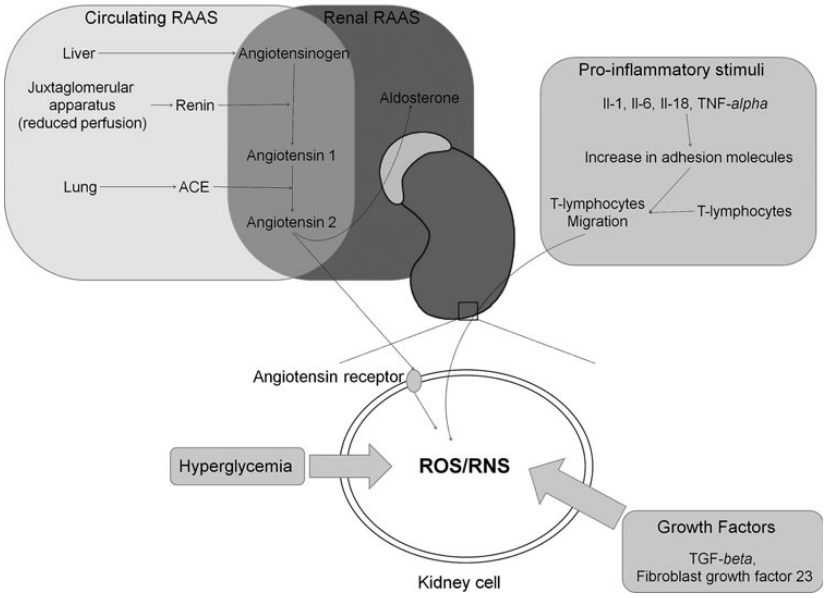


FIG. 3. Triggers for oxidative/nitrosative stress in kidney disease. A variety of factors external and internal to kidney can trigger oxidative/nitrosative effects within kidney. These include the circulating/renal RAAS systems, pro-inflammatory stimuli, hyperglycemia, and growth factors.

is thought that a local renal RAAS contributes to hypertension (52). RAAS inhibitors have protective effects on kidney in diabetics, most probably through reduction of blood pressure (85).

These triggers activate proinflammatory, profibrotic, and proapoptotic pathways, with consequent changes at the level of the proteome, the total protein complement of kidney cells and related tissues. Thus, proteomics has become a popular approach to explore these processes further (reviewed in Refs. 10, 32, 59, 64). Since proteins absorb the bulk of ROS/RNS, redox proteomics in particular offer opportunities to follow redox-mediated post-translational modification of proteins (73–75). A fundamental difference between the traditional proteomics and redox proteomic approaches is that reversible redox modifications can alter protein activity even in cases where the relative protein abundance may not necessarily change. Given the importance of oxidative stress in kidney pathology, it is intriguing that redox proteomics have not been applied more extensively to its investigation.

Kidney Proteomics

A range of experimental approaches are used in proteomic studies. These include two-dimensional electrophoresis (2DE) that combines isoelectric focusing with orthogonal sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Individual proteins are separated from complex mixtures based on differing isolectric point (pI) and M_r (32, 73–75). When combined with immunodetection, it is often possible to find redox modifications to proteins in 2DE separations such as carbonylation and nitration (73, 75). Quantitative data can be obtained from 2DE using difference gel electrophoresis (DIGE) with DeCyder software (77). This has allowed differential identification of several proteins in animal models (6) and in human urine (56). Mass spectrometry (MS) approaches have also proved useful in research on kidney diseases (53) (Fig. 4). As with DIGE, quantitative MS is increasingly being used (80). This may exploit labeling (either chemical or metabolic) or label-free methods. Labeling approaches include isotope-coded affinity tags (ICAT), stable-isotope labeling of amino acids in cell culture (SILAC), and isobaric tags for relative and absolute quantification (iTRAQ). Thiol-specific ICAT reagents have been exploited to measure oxidation of cysteines in the total proteome (18). SILAC offers a potentially powerful tool in the quantification of redox proteomic targets and their modification in kidney samples (35). iTRAQ has only yet been used in a few studies such as alteration of the phosphoproteome in the epithelial-to-mesenchymal transition induced in HK-2 cells by transcription growth factor-beta-1 (11). As of yet, ICAT is a relatively under-exploited approach in kidney proteomics.

Once potential redox-modified proteins have been detected within samples by a discovery approach, they can be validated and quantified by selective reaction monitoring (SRM) (13). This selects proteotypic peptides of the protein of interest and specific fragment ions (or transitions) upon fragmentation of the parent ion. This is the most sensitive proteomic quantification method available and detects low-abundance proteins with a dynamic range of up to 4–5 fold (13). SRM has potential in the quantification of redox protein biomarkers but is not ideal for the quantification of a specific redox modification within proteins. Peptides containing redox modifications are not suitable as proteotypic peptides as they can decrease or eliminate the signal of the corresponding precursor ion. Nevertheless, this approach has potential for quantification of redox-responsive proteins.

In addition to the two "workhorse techniques" of classical proteomics (2DE and MS), novel selection/screening methods are being used, sometimes in conjunction with MS. Surface-enhanced laser desorption ionization (SELDI) uses

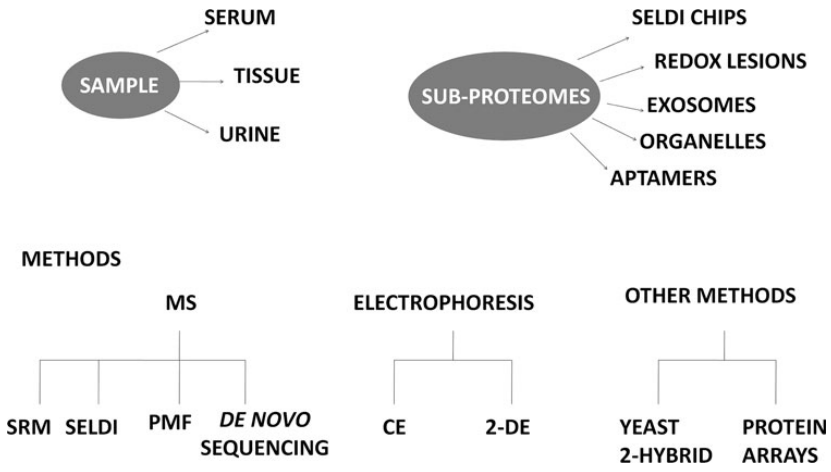


FIG. 4. Work-flows in kidney proteomics. Kidney proteomics studies have focused on kidney tissue, plasma/serum, or urine of patients or animal models. These can be further subfractionated to subproteomes to simplify sample complexity. Selected MS, electrophoresi,s and other methods are listed.

functionalized chips (carrying affinity or ion exchange groups) to select subproteomes. Large numbers of samples can be analyzed quickly, which is ideal for screening of clinical samples. For example, this has been used in studies of interstitial fluid of kidney biopsy material (41). A disadvantage of SELDI is that it only allows M_r determination rather than specific protein identifications. A novel recent selection approach has involved the use of aptamers, random libraries of single-stranded oligonucleotides which can adopt an enormous number of structural conformations with protein-binding properties. This selected 60 proteins in the serum of patients with chronic kidney disease, many of which were low M_r proteins often observed in this pathology (22). Fractionation of serum samples on magnetic beads allows MALDI-MS analysis (67). Another approach is to use protein microarrays; a recent study of serum revealed angiotensinogen and PRKRIP1 as novel autoantibody targets in kidney (9).

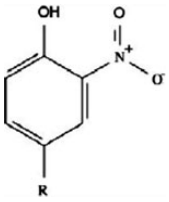
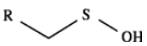
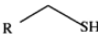
Sample preparation is a key aspect of proteomics experiments and it is increasingly evident that this step needs to be standardized for discovery of reproducible biomarkers (80). Traditional diagnosis of kidney disease often depends on performance of a biopsy. However, this may not always be possible for clinical (e.g., obesity, severe hypertension, or bleeding disorders) or ethical reasons and could result in a poorly defined sample. Proteins are well represented amongst extant biomarkers of chronic kidney disease (17). Therefore, many proteomic studies relevant to kidney disease focus on plasma or serum, tissues (e.g., from animal models), and urine (66). In particular, there have been major developments in studies of urine because of the sample quantity, possibility of repeat sampling, and its noninvasive nature (10, 48). A public domain proteomic database for kidney-associated proteomes is curated by an international consortium (Human Kidney and Urine Proteome Project, HKUPP; http://www.hkupp.org/) and this includes standardized protocols for urine sample preparation. The reader is also referred to the website of the European Consortium on kidney and urine proteomics (http://www.eurokup.org). Such databases may eventually facilitate comparisons between proteomes of healthy and diseased kidneys.

Studies on Human Kidney Tissue

Difficulties inherent in human kidney tissue proteomics have been eloquently described (59). Samples must be obtained invasively (e.g., by percutaneous biopsy) which yields small amounts of material, making comparison with healthy tissue ethically and logistically problematic. Such biopsies may not faithfully reflect the differing compartments/cell types in the composition of kidney. It has recently been pointed out that proteomic study of renal structures and compartments offer many opportunities for insights to kidney pathologies (10). A key technique is microdissection of defined kidney compartments such as glomeruli (87). This approach successfully identified autoimmune antigens in patients suffering from membranous glomerulonephritis, and suggested that oxidative stress in this pathology may drive superoxide dismutase 2 expression (58). An alternative is to prepare glomeruli by sieving. Using tissue from healthy cortex of four patients undergoing nephrectomy for renal tumors (renal cell carcinoma), this approach allowed proteome profiling by PDQuest™ image analysis and protein identification by 2DE and MS (88). Including novel pre-fractionation by one dimensional electrophoresis and solution-phase isoelectric focusing coupled with SDS PAGE, these workers identified more than 3500 proteins with at least two tryptic peptides (28). Using "microsieving," it has even proved possible to isolate whole glomeruli from single needle biopsy samples suitable for protein profiling (33).

Podocytes are kidney epithelial cells that wrap around the capillaries of the glomerulus which have been implicated in the early stages of kidney disease. MS analyses on five separate MS instruments identified a profile of 332 distinct proteins in a 1 μg biopsy sample of human glomerulus, including podocyte-essential proteins such as podocin and nephrin (89). Expression profiling of podocytes exposed to high glucose as a model for diabetic nephropathy identified up-/downregulated genes including biomarkers of chronic kidney disease such as neutrophil gelatinase-associated lipocalin (26). A recent proteomic study of cultured podocytes revealed that RAAS activation caused downregulation of the antioxidant

Table 1. Redox Proteomic Studies of Kidney Disease

Modification	*Sample*	*Proteins identified (unique*)*	*Method*	*References*
3NT 	Plasma and hemodialysates from uremic patients	15 (7)	2DE followed by WB for 3NT	57
	Kidney medulla of spontaneously hypertensive rats (SHR)	20 (13)	2DE followed by WB for 3NT	73
	Kidney mitochondria of diabetic rats (diet induced)	none	2DE followed by WB for 3NT	30
	Plasma of long term hemodialysis patients	1 (0)	Immunoprecipitation of 3NT containing proteins followed by WB for specific proteins	45
Sulfenic acid 	Kidney medulla of SHR	39 (36)	Biotin labeling of sulfenic acids, 2DE followed by WB	76
Free thiols 	Plasma of glomerulosclerosis patients	26 (15)	Labeling of free thiols with cyanines followed by DIGE	8
Carbonylation 	Kidney medulla of SHR	11 (3)	DNPH labeling of protein carbonyls, 2DE followed by WB	74
	Plasma of healthy donors	†	Biotin labelling of protein carbonyls followed by affinity chromatography and MS analysis	40

*Denotes proteins only identified in one study presented in this table.
†As only healthy subjects were used in this study, no protein can be associated to kidney disease. Carbonylated proteins, as well as many other OS-related PTMs, were present in the subjects, many of kidney origin.

protein peroxiredoxin 2. This led to production of ROS, protein oxidation, inhibition of the Akt pathway, and onset of apoptosis (25). This is consistent with the observation in biopsy samples that SOD 2 is induced by oxidative stress in membranous nephropathy (58).

Studies on Blood Plasma/Serum

Plasma and serum samples derived from patient blood provide a less invasive sample source for proteomics that is also amenable to re-sampling. A particular problem with blood-derived samples is the broad dynamic range of blood protein concentration, with some proteins being very abundant whilst others are rare. The most abundant proteins can be depleted by immunoaffinity fractionation. After immunodepletion for the six most abundant proteins (albumin, transferrin, IgG, IgA, haptoglobulin, and antitrypsin), proteomic comparison of serum from diabetic nephropathy identified two biomarkers (selenium-containing extracellular glutathione peroxidase and apolipoprotein E) which were downregulated in this pathology (31). Both of these proteins are implicated in antioxidant defense (31). An alternative to immunodepletion involves use of a library of hexameric peptides that allows selection of the total proteome, including very low abundance proteins. This is based on creating at random an enormous repertoire of protein-binding sites which makes possible identification of a greater number of urinary proteins. Chronic kidney disease patients have a higher risk of premature atherosclerosis. An MS-based approach combining capillary electrophoresis (CE) coupled to MS analysis of serum of chronic kidney disease patients identified protein biomarkers for cardiovascular disease (62). 2DE of non-immunodepleted serum also identified four proteins (alpha-1-microglobulin, apolipoprotein A-IV, gamma-fibrinogen, and haptoglobin) associated with atherosclerosis in patients with chronic kidney disease (39). Chronic kidney disease is a common feature in patients after liver transplant, and proteomic analysis of serum has been reported using a commercial ELISA-based technology [multi-analyte panels (MAPs)] of 188 serum proteins and a kidney MAP of 14 kidney-associated proteins, which was predictive of later onset of disease, even in patients with only mildly diminished glomerular filtration rate at the time of transplant (36). A protein microarray study of serum recently discovered angiotensinogen and PRKRIP1 as novel autoantibody targets (9).

Advanced oxidation protein products are oxidized variants mainly of albumin and fibrinogen that have been identified in plasma/serum and are elevated in chronic kidney disease (63). More than 70% of the free radical-trapping capacity of serum is attributed to albumin. The oxidation status of serum proteins correlates with systemic inflammation during chronic kidney disease (43). Redox-induced post-translational modifications (PTMs) of protein residues include thiol oxidation and carbonylation (formation of aldehyde or ketone

groups in side-chains) (43, 82). Redox proteomics approaches can identify oxidized proteins in serum/plasma. An elegant approach to profiling of carbonylated proteins in human plasma involved trapping this subproteome on hydrazine-biotin with avidin selection (40). A combination of MALDI-TOF-TOF and electrospray-tandem MS identified 65 carbonylated proteins, many of which were from kidney. Interestingly, several of these proteins carried multiple redox lesions and oxidation of methionine was the most commonly found modification. In studies of this type, kidney is consistently one of the largest contributors of plasma proteins (40).

Another category of redox modification found in proteins (Table 1) is nitration, in particular, modification of tyrosine to 3-nitrotyrosine (73, 76). Increased 3-nitrotyrosine in both chronic and end-stage kidney disease patients was found in serum proteins by both immunoblotting and LC-tandem MS (57). Cysteine, the second least abundant residue in proteins, is also subject to extensive redox modification and (with methionine) contributes the bulk of antioxidant activity of human serum albumin. Cysteine can exist in a variety of disulfide-bridged (e.g., homocysteinylated, glutathionylated), nitrosylated, and other oxidized variants (e.g., sulfenic, sulfinic, and sulfonic acids) (82). Serum proteins such as low density lipoproteins have been reported to have elevated homocysteinylation in chronic kidney disease patients (90), whilst irreversible oxidation of Cys-34 of serum albumin and increase in albumin carbonylation correlates with increasing stage of chronic kidney disease (43). Given the availability of ready methods for detecting redox lesions at methionine (40) and thiols (75), it is surprising how few studies exploit such approaches in serum proteins. Iodoacetamido-substituted cyanine labels are a possible route to profiling of cysteine-containing proteins in serum (8).

Studies on Urine

Kidney disease is commonly diagnosed by albuminuria, reflecting diminished retention of protein by the kidney. Urine therefore offers potential as "readout" for diminished kidney function, which is also available noninvasively and may be repetitively sampled. Recent proteomic research in kidney disease has accordingly focused on urine (reviewed in Refs. 2, 46). In particular, small peptides, the peptidome, consisting of peptides largely of extracellular matrix origin are informative about the onset of chronic kidney disease (2). Considerable progress has been made in standardizing sample preparation and data analysis protocols for the kidney peptidome and proteome (44). Comparison of the plasma and urine proteomes revealed that the average M_r of the urine proteome is indeed smaller than that of plasma (27). This arises from the filtration and secretion activities of kidney plus proteins secreted or shed from downstream glands and the urinary tract. The analysis identified 2280 plasma proteins that were blocked by kidney, 394 proteins were common to plasma and urine (suggesting kidney allows them to pass through), while 1128 proteins were unique to urine, including proteins secreted/shed by kidney, downstream glands, and the urinary tract (27). Abundant plasma proteins can enter the urine once the glomerular filtration barrier is altered in chronic kidney disease, making fibrosis-associated proteins difficult to detect (32).

A significant body of literature has arisen on exosomes in urine (79). These are small (40–100 nm) endocytic vesicles originating from a range of cell types and secreted into body fluids (including urine) or the extracellular space. Exosomes carry proteomes reflecting their cells of origin and provide a ready means of profiling urine in a range of kidney pathologies (79). A compendium of exosome proteins is now available online (http:/exocarta.ludwig.edu.au; 42).

As with serum/plasma, virtually no systematic investigation of redox protein variants has been performed on the proteomes of either urine or exosomes. Given that these sources frequently contain proteolyzed and otherwise modified proteins, it would seem that redox PTMs would offer a fruitful comparator across samples from differing kidney pathologies.

Studies in Animal Models

An alternative to human renal tissue is offered by animal models that are widely used in kidney disease research

Table 2. Selected Animal Models Used in Proteomic Research in Kidney Disease

Pathology	*Model*	*Cause*	*References*
Chronic kidney disease	Spontaneously hypertensive rat	Spontaneous	73–75
Chronic hypoxia	Rat	Surgical (stenosis)	65
Diabetes	Rat	Streptozotocin-induced	6, 61
Acidosis	Rat	Induced with ammonium chloride via drinking water	14
Passive Heymann nephritis	Rat	Induced with antibodies	50
Nephrectomy	Rat	Surgical	86
Ischemic-reperfusion injury	Rat	Surgical	16
Sepsis	Rat	Surgical (cecal ligation and puncture)	24
Adriamycin-nephropathy	Rat	Induced with adriamycin	81
Membrane proliferative glomerulonephritis	Mouse	Knockout (complement regulatory protein factor H (FH-/-)	7
Hypokalemic nephropathy	Mouse	Diet	70
Diabetes	Mouse	Knockout (db/db diabetic)	4
Immune nephritis	Mouse	Induced with antibodies to glomerular basement membrane	84
Nephritis	Dog	Inherited	47

(reviewed in Ref. 32). These often allow detection of events on a relatively short time scale that might take years to develop in humans. In general, animal models are genetic, may have arisen spontaneously, or else are caused surgically or by drug treatment (Table 2). The spontaneously hypertensive rat (SHR), originally developed from Wistar rats by selective breeding, spontaneously displays hypertension emerging in weeks 12–14 and persisting for more than a month (54). The SHR lifespan (1.5–2.5 years) is significantly less than that of normotensive rats (2.5–3 years), but this difference makes life-long studies feasible in a practical timeframe. SHRs do not absolutely mirror hypertension in humans (e.g., onset is in the equivalent of young adulthood in SHRs but in middle age in humans). Nevertheless, SHRs are a sufficiently good animal model for the testing of novel antihypertensives (37). Redox proteomics studies have been performed in SHR kidney tissues, including identification of kidney proteins containing 3-nitrotyrosine (73), sulfenic acid (75), and carbonylation (74).

Other animal models for kidney disease research in which proteomics have featured include diabetic rats (6, 61), acidotic rats (14), passive Heymann nephritis rats (50), nephrectomized rats (86), ischemic-reperfusion injury in rat kidneys (16), sepsis in rats (24), stenosis in rats (65), genetically-modified mice (7), mice with hypokalemic nephropathy (70), diabetic mice (4), mice with immune nephritis (84), and dogs with inherited nephropathy (47). These studies often point to oxidative stress as a key feature of chronic kidney disease and agree well with knowledge of disease progression in humans. For example, downregulation of superoxide dismutases (58) and upregulation of the NAD(P)H oxidase (NOX) family (19), a ubiquitous source of superoxide, are found both in animal models and humans. An elegant illustration of the power of animal models in the study of kidney pathology is the overexpression of catalase along with angiotensinogen in a double-transgenic mouse (21). Catalase overexpression in renal proximal tubules resulted both in decreased oxidative stress and decreased fibrosis, albuminuria, and hypertension (21).

Future Directions

Proteomics is now an established route to study of important kidney pathologies (10, 32, 59, 64). Some proteins are oxidized due to oxidative/nitrosative stress associated with kidney disease (43, 57). However, this review documents that redox proteomics is a heretofore relatively underutilized approach which nonetheless has enormous potential to offer insights to kidney pathology. Oxidative stress is a key factor in many kidney pathologies, especially chronic kidney disease (15, 60), and reversal of oxidative stress often alleviates clinical manifestations such as hypertension (21, 37). Interception of routes to oxidative stress has been suggested as a novel drug therapy approach for chronic kidney disease (68), and beneficial results have recently been found in a rat diabetic nephropathy model with the oral hypoglycemic drugs metformin (3) and pioglitazone (71). These drugs also have beneficial effects in human diabetics (29). As redox proteomics now allows routine fingerprinting of oxidative PTMs, there is potential here for early-stage detection of disease onset in complex samples such as plasma/serum and urine. Kidney proteins are particularly well represented in serum/plasma proteomes (40), and appearance of proteins in urine is an early indicator of chronic kidney disease, making urine a key sample source for kidney proteomics (2, 46). Amongst proteins found to be modulated by pathology within kidney tissues, antioxidant proteins are often prominent (25). These findings suggest that analyses of urine and exosome proteomes may be especially informative in the future.

A number of recent technical developments could underpin systematic redox proteomic study of clinical samples such as plasma/serum and urine. Standardized protocols for sample preparation, especially from urine, are now available (see: http://www.hkupp.org/; http://www.eurokup.org). Selection of subproteomes such as those of exosomes (42, 79) or carrying specific PTMs such as carbonylation (40) can be used to simplify complex samples, select for low-abundance proteins, or identify modified variants within separations (Fig. 5). There is also growing interest in gel-free proteomics approaches including quantitative MS (e.g., ICAT, SRM) that have not yet been applied extensively to kidney proteomics. Moreover, availability of new high-speed/high-resolution MS platforms such as the Orbitrap Elite ETD and Triple TOF 5600, when combined with two-dimensional liquid chromatography and nano-HPLC systems, make feasible high

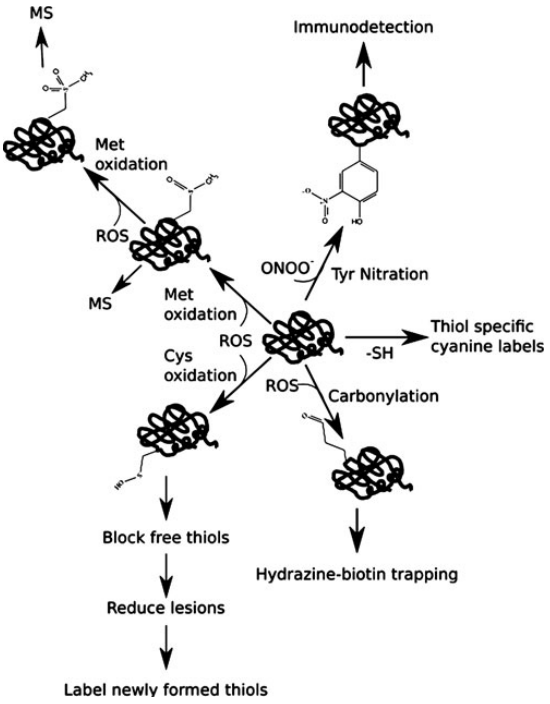


FIG. 5. Selection and identification options for oxidatively-modified proteins in redox proteomics. Redox lesions can provide means for readily enriching or detecting subproteomes of modified proteins. Relatively common modifications include carbonylation or methionine oxidation to sulfoxides/sulfones. Rarer modifications include tyrosine nitration and formation of sulfenic acid from cysteine thiols.

sample throughput and accurate PTM identification. This would make feasible mapping of redox lesions to specific residues within target proteins. Thus, redox proteomics offers a technologically feasible route to more detailed characterization of complex proteomes in kidney pathology with potential for insight to disease onset (including novel early biomarkers), assessing new drugs, understanding biochemical mechanisms, and fingerprinting of variation within patient populations.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ACE = angiotensin-converting enzyme
CE = capillary electrophoresis
2DE = two-dimensional electrophoresis
DIGE = difference gel electrophoresis
ELISA = enzyme-linked immunosorbent assay
HKUPP = Human Kidney and Urine Proteome Project
ICAT = isotope-coded affinity tags
iTRAQ = isobaric tags for relative and absolute quantification
MALDI-TOF = matrix-assisted laser desorption ionization-time of flight
MAPs = multi-analyte panels
MS = mass spectrometry
NOX = NAD(P)H oxidase
pI = isolectric point
PTM = post-translational modification
RAAS = renin-angiotensin-aldosterone system
RNS = reactive nitrogen species
ROS = reactive oxygen species
SDS PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI = surface-enhanced laser desorption ionization
SHR = spontaneously hypertensive rat
SILAC = stable-isotope labeling of amino acids in cell culture
SRM = selective reaction monitoring
WB = Western blotting

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A proteomic evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine mussels (*Mytilus* spp.): evidence for chronic sublethal effects on stress-response proteins

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Human pharmaceuticals (e.g. the lipid regulator gemfibrozil and the non-steroidal anti-inflammatory drug diclofenac) are an emerging environmental threat in the aquatic environment. This study aimed to evaluate sublethal effects of these two commonly found pharmaceuticals on the protein profiles of marine mussels (*Mytilus* spp.). *Mytilus* spp. was exposed to environmentally relevant and elevated concentrations (1 and 1000 μg/l respectively) of both drugs for 14 days. In addition, mussels were maintained for seven days post treatment to examine the potential of blue mussels to recover from such an exposure. Differential protein expression signatures (PES) in the digestive gland of mussels were obtained using two-dimensional gel electrophoresis after 7, 14, and 21 days of exposure. Twelve spots were significantly increased or decreased by gemfibrozil and/or diclofenac, seven of which were successfully identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. These proteins were involved in energy metabolism, oxidative stress response, protein folding, and immune responses. Changes in the PES over time suggested that mussels were still experiencing oxidative stress for up to seven days post exposure. In addition, a suite of biomarkers comprising glutathione transferase, lipid peroxidation, and DNA damage were studied. An oxidative stress response was confirmed by biomarker responses. To our knowledge, this is the first investigation using proteomics to assess the potential effects of human pharmaceuticals on a non-target species in an environmentally-relevant model. The successful application of this proteomic approach supports its potential use in pollution biomonitoring and highlights its ability to aid in the discovery of new biomarkers. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Diclofenac; gemfibrozil; *Mytilus* spp.; biomarkers; proteomics

Introduction

In recent decades the amount of human and veterinary pharmaceuticals produced, consumed, and ultimately released into the aquatic environment has consistently increased.[1,2] Pharmaceutical substances are designed for a specific mode of action and often remain biologically active during their passage through an organism's body.[1] While therapeutically active, many drugs are resistant to degradation.[2] Therefore pharmaceuticals and their metabolites often have the ability to bioconcentrate and bioaccumulate in the environment and could potentially pose a toxic threat to a wide range of non-target organisms.[2] In addition to their persistence, these novel contaminants are being constantly released into the environment, mainly through the release of effluents from municipal wastewater treatment plants.[3,4] Concentrations of individual pharmaceuticals in marine surface waters, rivers, and lakes throughout North America, continental Europe, the UK, and Ireland have been found to be in the high ng/l to the low μg/l range.[3,5–7] In particular, two commonly used drugs, the non-steroidal anti-inflammatory (NSAID) drug diclofenac and the lipid-lowering agent gemfibrozil (GEM), are often found in receiving waters worldwide, including Ireland.[5–7]

With its analgesic, anti-inflammatory, and antipyretic properties, diclofenac is commonly prescribed to treat acute and chronic pain as well as inflammatory conditions.[8] Its mode of action includes the inhibition of prostaglandin synthesis by inhibiting both cyclooxygenase-1 and −2 (COX-1 and COX-2).[8] In wildlife, diclofenac has been shown to cause lethal effects: vultures in Asia which scavenged on diclofenac-treated livestock died from acute renal failure.[1,9] In addition, studies have demonstrated sublethal adverse

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effects and bioaccumulation potential in aquatic organisms, such as fish, under environmentally relevant concentrations.[10–12]

GEM, a lipid-lowering fibrate, is used by humans to decrease plasma triglyceride and cholesterol concentrations. In general, fibrates induce fatty acid uptake, which are then converted to acyl-CoA derivatives and finally catabolized by β-oxidation.[13] These actions are mediated by the peroxisome proliferators-activated receptors (PPARs). GEM can induce peroxisome proliferation by binding to the PPAR α, β and γ.[13] In addition, GEM has potential to bioaccumulate and was found to reduce the plasma testosterone concentration in male goldfish.[14,15]

The widespread use of pharmaceutical products and their continuous release into the aquatic environment means that there is a need for assessment of their possible ecotoxicological effects on non-target organisms.[1] Currently, standard toxicity tests and a wide range of molecular biomarkers are used. Previously, biomarkers such as glutathione transferase (GST) activity, metallothionein, lipid peroxidation (LPO), DNA damage, and vitellin-like proteins have demonstrated that exposure to both pharmaceuticals significantly affects detoxification and defense systems of blue mussels.[16] However, these tests are either based on short-term acute toxicity, which is only relevant when accidental discharge of the drug occurs,[2] or cover only particular aspects of a general stress response, making them unsuitable to identify potential effects of pharmaceuticals. Furthermore, many biomarkers are influenced by multiple confounding factors, which may limit their transfer from laboratory to field studies.[17] With a proteomic approach, a more detailed understanding of the toxicology of the compounds can potentially be achieved since a relatively large number of proteins can be analyzed simultaneously.[18] Changes in the protein expression signature (PES) of an organism or tissue can help identify specific responses to a stressor, such as pharmaceuticals.[19] This could help not only in the understanding of the mechanisms of action, but also in the recognition of novel, specific biomarkers. In the past, various laboratory and field studies have demonstrated successful application of a proteomic approach in a range of organisms, including bivalves.[20–24] Marine bivalves, such as blue mussels (*Mytilus* spp.) are a popular bioindicator species, widely used in environmental monitoring programmes such as the Mussel Watch Programme.[25] Due to their sessile life style, worldwide distribution and filter-feeding behaviour, they are a widely used model bioindicator species for pollution in estuaries and marine waters.[26]

This study aimed to investigate the potential effects of two commonly found human pharmaceuticals, diclofenac and GEM, on marine mussels (*Mytilus* spp.) exposed for 14 days to environmentally relevant and elevated concentrations of the compounds (1 μg/l and 1000 μg/l, respectively). In addition, mussels were maintained for an extra week to investigate the potential for recovery from exposure to pharmaceuticals. Chronic sublethal effects in the digestive gland of *Mytilus* spp. were examined by investigating PES as well as by the determination of three biomarkers. This work could give insights into mechanisms involved in the response of aquatic non-target organisms to these two drugs. To our knowledge, this is the first study using proteomics to assess chronic sublethal effects of diclofenac and gemfibrozil in blue mussels.

Material and methods

Sampling and experimental design

Blue mussels (*Mytilus* spp.) were collected in June 2010 from a pristine site in the west of Ireland (Lettermullan, Co. Galway; 53°14′38″N 9°43′34″W). The coastline of Ireland is included in the large mussel hybrid zone of *M. edulis* and *M. galloprovincialis*. This zone contains a mixture of pure, hybrid and introgressed individuals.[27] Since there is no single morphological characteristic that can be reliably used to separate this mixed population, we were not able to classify the exact species used in this study. However, all mussels sampled came from the same population from a specific location. Mussels (4–5 cm) were transported to the laboratory, cleaned and acclimatized for 7 days in dechlorinated artificial sea water (ASW) at 13 °C (± 1 °C) with a 12-h light/dark regime. Water temperature (in °C), pH, oxygen and nutrients (ammonia (NH_4^+ in mg/l), nitrate (NO_3 in mg/l), and nitrite (NO_2 in mg/l)) were measured at the study site and twice a week during exposures. Mussels were fed daily with a commercial solution containing *Isochrysis* algae at the recommended 2% maximum feed conversion rate during acclimatization and exposure. Individuals were exposed to two concentrations of GEM and diclofenac (1 μg/l and 1000 μg/l, respectively) under semi-static conditions with daily water change. The drugs were dissolved in dimethyl sulphoxide (DMSO) and added to the tanks after each water change, with a final DMSO concentration of 0.001% (*v/v*). Mussels in control (ASW) and solvent control (ASW + 0.001% DMSO) tanks were included in the experimental design. Separate exposures to either drug were run simultaneously. In order to minimize exposure *via* food, mussels were fed 3 h before the water was changed and fresh contaminant added. To evaluate the potential change in concentration of the compound over a 24-h period, the actual concentration of each compound was monitored on three separate occasions (days 7, 10, and 14) during the experiment. To do this, 500-ml water samples were taken at 0 and 24 h for each concentration of each drug. All treatments, as well as control and solvent control groups, consisted of 3 replicate tanks. The exposure lasted for 14 days; additionally mussels were left for another 7 days without any treatment. After 7, 14, and 21 days, the visceral mass was dissected and frozen at −80 °C for further analysis. To determine the Fulton condition factor (CF = ((W/L*10)) the total weight (W in g) and the shell length (L in cm) of each mussel was determined.

Tissue preparation

For each of the three sampling times (days 7, 14, 21), there were four treatments (control, solvent control, concentration 1 (1 μg/L), concentration 2 (1000 μg/L)) with each treatment containing three treatment tanks as replicates. Three mussels were taken from each treatment tank, the digestive gland dissected over ice and pooled together, resulting in a sample size of 72. The samples were homogenized in a Tris–HCl buffer containing 10 mM Tris–HCl, 0.5 M sucrose, 0.15 M potassium chloride (KCl), 1 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM Phenylmethanesulfonyl fluoride (PMSF) with a pH of 7.2 at a weight: volume ratio of 1:4. Subsamples of the homogenate were frozen at −80 °C for later analysis of lipid peroxidation (LPO) and DNA damage. The remaining homogenate was centrifuged at 15 000 g for 1 h at 4 °C and the supernatant (S15) was separated from the pellet, frozen at −80 °C until required. These were used to analyze the soluble protein fraction by two-dimensional electrophoresis (2-DE) and to determine glutathione transferase (GST) activity. The total protein content in the supernatant and homogenate was evaluated by the Bradford method.[28]

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Biomarker assessment

Glutathione transferase (GST) activity

GST activity was determined using the method of Habig *et al.*[29] Fifty microlitres of S15 from digestive gland was added to 200 µl of 1 mM reduced glutathione (GSH) and 200 µl of 1 mM 1-chloro-2, 4-dinitrobenzene in a buffer of 10 mM Hepes-NaOH containing 125 mM NaCl, pH 7.2. Absorbance at 340 nm was read spectrophotometrically after 0, 1, 5, 10, and 20 min and data were expressed as µmoles GSH transferase activity/ min/ mg protein.

Lipid peroxidation (LPO)

Lipid peroxidation was measured by the thiobarbituric acid method adapted from Wills.[30] One hundred and fifty microlitres of digestive gland homogenate was added to 300 µl 10% trichloroacetic acid (TCA) containing 1 mM iron sulfate ($FeSO_4$) and 150 µl 0.67% thiobarbituric acid (TBA). After heating for 10 min, samples were centrifuged for 5 s at 10 000 g to remove precipitate. Two hundred microlitres of sample was added to a black 96-well microtitre plate and fluorescence was measured at 515 excitation/ 545 nm emission. Blanks and standard solutions of tetramethoxypropane were prepared in homogenate buffer. Thiobarbituric acid reacting substances (TBARS) were expressed as µmoles/mg protein.

DNA damage (strand break)

DNA damage was assessed by adapting the method of Olive.[31] Twenty five microlitres of digestive gland extract was added to 200 µl 2% sodium dodecyl sulfate (SDS) containing 10 mM EDTA, 10 mM Tris–base and 40 mM sodium hydroxide (NaOH). After 1 min, 200 µl of 0.12 M potassium chloride (KCl) was added and the solution was heated for 10 min at 60 °C. After 30 min cooling at 4 °C, samples were centrifuged at 8000 g for 5 min at 4 °C. Fifty microlitres homogenate was added to 150 µl of Hoescht (bisBenzimide) containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris–acetate, pH 8.5–9. After 5 min mixing fluorescence at 360 nm excitation/450 nm, emission was determined. Homogenization buffer was used as a blank. Salmon sperm DNA standards (Sigma Aldrich) were added to a buffer solution containing 50 mM Tris–acetate and 1 mM EDTA, pH 8, and the formation of DNA strand breaks were expressed as µg/mg protein.

Proteomic analyses

Two-dimensional gel electrophoresis (2-DE) and staining

A volume of S15 containing 85 µg protein was precipitated with a final concentration of 10% TCA and centrifuged at 11 000 g for 3 min at 4 °C. Resultant protein pellets were resuspended in 40 µl water and precipitated with ice-cold acetone to wash away traces of TCA. Samples were then centrifuged at 11 000 g for 3 min at 4 °C. The protein pellet was resuspended in 125 µl rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1.2% bis (2-hydroxyethyl)-disulfide, 4% ampholytes (3–10 for IEF, GE Healthcare) and a trace of bromphenol blue) prior to rehydration of 7 cm IPG strips, pH 3–10 (GE Healthcare), for 18 h using an Ettan IPGphor III machine (Amersham Bioscience) according to the manufacturer's guidelines. Focused strips were equilibrated in 2% D/L-dithiothreitol for 20 min followed by 2.5% iodoacetamide for 20 min, both in equilibration buffer (6 M urea, 2% SDS, 20% glycerol and 0.375 M tris-buffer, pH 8.8). The equilibrated strips were placed on top of a 12% SDS-PAGE gel and proteins were separated. Protein spots were stained with colloidal coomassie.[32] The stained gels were then scanned using an Alpha Innotech scanner. To take biological variation into account three biological replicates were used (a total of 72 gels).

Protein spot analysis and in-gel digestion of proteins from 2-DE gels

Progenesis SameSpot proteomic analysis software (Non Linear Dynamics) was used to identify significant changes in protein spot intensity. Statistically significant different spots were identified according to their alteration in response to each treatment (i.e. GEM or diclofenac) at each exposure time (days 7, 14, or 21). Alterations were considered statistically significant when $p \leq 0.05$ according to ANOVA and a fold change of ≥ 1.5 relative to both control and solvent control was observed. In addition to statistical significance, principal component analysis (PCA- performed using all the spots present on the gel) was used to choose among significantly different spots for protein identification, keeping only the spots that contributed most to the difference between treatment and control.

Selected spots were manually excised using a clean scalpel and used for in-gel digestion with trypsin according to PO 25MS. The extracted peptides were loaded onto a R2 micro column (RP-C18 equivalent) where they were desalted, concentrated, and eluted directly onto a MALDI plate using α-ciano-4-hydroxycinamic acid (5 mg/ml, CHCA) as the matrix solution in 50% acetonitrile and 5% formic acid. Mass spectra of the peptides were acquired with positive reflectron MS and MS/MS modes using a MALDI-TOF/TOF MS instrument (4800 plus MALDI TOF/TOF analyzer). The collected MS and MS/MS spectra were analyzed in combined mode by using the Mascot search engine and the NCBI database restricted to 50 ppm peptide mass tolerance and no taxonomy restrictions (PO 25MS).

Chemical analyses

Methods for extraction and analysis of GEM and diclofenac were adapted from those described by Lacey *et al.*[6] Briefly, water samples were first filtered through Whatman glass fibre filters (1.2 µm) and adjusted to pH 4 using sulphuric acid. Pharmaceuticals were extracted from water samples using Phenomenex Strata-X solid phase extraction cartridges (200 mg, 6 ml). After cartridge conditioning, samples (500 ml) were loaded, rinsed with 6 ml water, dried for 30 min, eluted with 9 ml ethyl acetate/acetone (50:50) and reconstituted in 0.25 ml of 13 mM ammonium acetate with 20% acetonitrile. Separations were carried out with an Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) using a Waters Sunfire C_{18} column (3.5 µm 150 x 2.1 mm I.D.). Gradient elution of 13 mM ammonium acetate with 20% acetonitrile (A) and 100% acetonitrile (B) was carried out at a flow rate of 0.3 ml/min over a total run time of 30 min including 15 min re-equilibration time. Sample injection volume was 10 µl. Mass spectrometry was performed using a Bruker Daltonics HCT ion trap MS with an electrospray ionization interface at atmospheric pressure (Bruker Daltonics, Coventry, UK). The LC-ESI-MS/MS system was controlled using Bruker Compass HyStar version 3.2. Multiple reaction monitoring (MRM) was employed for mass spectrometric analysis of GEM and diclofenac in negative mode.

Data analyses

For statistical analysis of the condition factor, nine individual mussels per treatment and time-point were taken. Data are expressed as mean ± standard deviation (stdev). A one-way ANOSIM using PRIMER (version 6) was performed to evaluate differences between the combination of treatment groups and sampling time.

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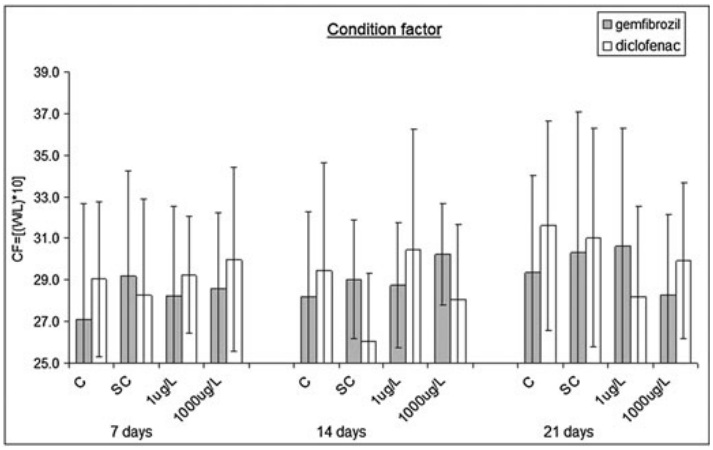

Figure 1. CF (mean ± stdev) of blue mussels (*Mytilus* spp.) exposed to 1 µg/l and 1000 µg/l gemfibrozil (grey bars) and diclofenac (white bars).

A two-way ANOVA with the factors time, treatment and interaction 'time x treatment' as variables using STATISTICA was used. The Shapiro-Wilk test and the Levene's test were used to assure normal distribution and homogeneity of the variances of the data. In cases where significance differences between the factors were detected, the Bonferroni test was used as a *post hoc* test. The level of significance differences were set at $p \leq 0.05$.

Results

Throughout the exposure time all mussels appeared to be in good health with their siphons fully extended indicating normal feeding by filtration. The CF of the mussels did not change significantly over the experimental time or between different treatments (Figure 1). During exposure, a mortality rate of only 0.84% was observed, with one fatality found after 9, 10, and 18 days and three fatalities after 19 days. These fatalities occurred in or after diclofenac exposure. Temperature, salinity, oxygen, pH and nutrients (NH_4^+, NO_3 and NO_2) were controlled over the experimental time. Average water temperature in each exposure was 13 °C (± 0.5) and 13.4 °C (± 1.5) for GEM and diclofenac exposure, respectively. Between the two simultaneous experiments a slight difference in water temperature was observed, however the largest difference in temperature was only 2.4 °C. No difference for the other parameters (salinity, pH, oxygen and nutrients (NH_4^+, NO_3 and NO_2)) were observed within each exposure or between both experiments.

Chemical analyses

Pharmaceutical concentrations were determined for each exposure tank (Table 1) at time 0 h, on three separate occasions during the experiment (days 7, 10, and 14) and after 24 h. No pharmaceutical residues were detected in any of the control or solvent control tanks. Recoveries for the solid-phase extraction of GEM and diclofenac in artificial seawater were 63% (RSD 6.8%, n = 6, spiking level 1 mg/l) and 44.7% (RSD 2.4%, n =6 spiking level 1 mg/l), respectively. For the environmentally relevant concentration of 1 µg/l GEM a reduction in actual concentration over 24 h was observed (Table 1). Similarly, a reduction of 1 µg/l diclofenac was observed at days 10 and 14. However, at day 7, the actual concentration measured after 24 h exposure was higher than the actual concentration measured at 0 h when the chemical was initially added to the tanks. This was also observed for the elevated concentration of 1000 µg/l of GEM and diclofenac, with the exception of day 14 where a reduction of 1000 µg/l diclofenac was observed.

Biomarker analyses

GST activity, a biomarker for stress,[33] was significantly increased at day 21, a week after GEM exposure. In addition, GST expression

Table 1. Measured concentrations of GEM and diclofenac. Water samples were taken three times during the experiment after adding the chemical to the water column (0 h) and after 24 h immediately before the water change. Concentrations are shown in µg/L as mean ± standard error

	7 days		10 days		14 days	
GEM	0 h	24 h	0 h	24 h	0 h	24 h
1 µg/L	0.59 ± 0.04	0.13 ± 0.05	1.00 ± 0.04	0.68 ± 0.04	0.75 ± 0.04	0.32 ± 0.04
1000 µg/L	290.43 ± 44.11	383.46 ± 42.87	270.79 ± 41.93	358.26 ± 40.73	251.07 ±42.22	357.76 ± 40.74
	7 days		10 days		14 days	
Diclofenac	0 h	24 h	0 h	24 h	0 h	24 h
1 µg/L	0.65 ± 0.07	0.87 ± 0.08	0.75 ± 0.06	0.60 ± 0.06	0.78 ± 0.06	0.65 ± 0.06
1000 µg/L	111.14 ± 73.68	241.14 ± 68.47	80.80 ± 69.21	409.99 ± 61.48	340.32 ± 62.76	102.06 ± 59.95

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was significantly increased in mussels previously exposed to 1 μg/l and 1000 μg/l of GEM as well as in the solvent control in comparison to control mussels at day 21 (Figure 2). However, no significant changes were observed for both biomarkers of damage, LPO and DNA damage, during GEM exposure (Figure 3). In contrast, GST expression was significantly decreased at day 21 after diclofenac exposure (Figure 4) with a significant decrease in mussels exposed to 1000 μg/l at day 14 in comparison with control and solvent control mussels. In addition, decreased levels of LPO after 21 days of diclofenac exposure were observed (Figure 5). Similarly, significantly lower DNA damage was measured at days 14 and 21 (Figure 6). Significantly lower DNA damage at day 14 was also measured in mussels exposed to 1 μg/l and 1000 μg/l diclofenac in comparison with solvent control. In contrast, significantly higher DNA damage was found in mussels exposed previously to 1000 μg/l diclofenac at day 21 when compared to control and solvent control animals.

Proteomic analyses

Proteins were separated in well-defined spots, as seen with colloidal coomassie staining (Figure 7). A total of 266 spots per 2-DE gel were analyzed using the Progenesis software.

In total, 12 spots were found to be significantly increased or decreased by diclofenac and/or GEM treatment (ANOVA $p \leq 0.05$). All spots showed a ≥ 1.5-fold change for at least one condition relative to control as well as grouping with the treatment in PCA. Four spots were found to be regulated specifically by diclofenac, one of which was up-regulated at both concentrations and over the full experimental time (Table 2). Two of three spots specifically modified by GEM exposure showed an up-regulation after 14 and 21 days of treatment. Five spots were shown to be significantly up and down-regulated by both pharmaceuticals.

Among the spots of interest, seven, including up- and down-regulated spots were successfully identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Table 3). Identified spots belonging to various functional groups could be related to general stress response, oxidative stress and protein folding. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; id: 0155), aconitase 1 (eID: 0005), and class 1 alcohol dehydrogenase (ADH), beta subunit (id: 0169) often implicated in oxidative stress responses were identified. The metabolic enzyme

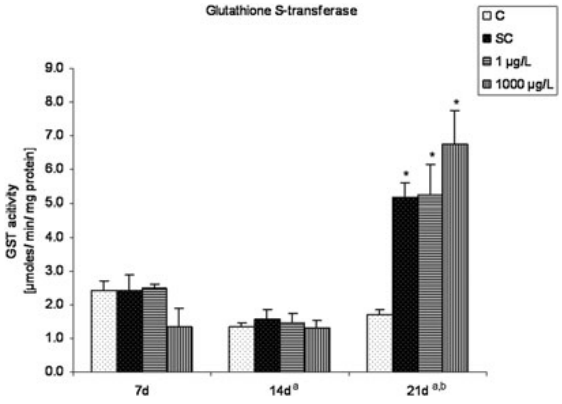

Figure 2. GST activity (mean ± stdev) of blue mussels exposed to 1 μg/l and 1000 μg/l GEM. Significance set at $p \leq 0.05$ with (a) indicating differences to day 7, (b) to day 14 and (*) significant to control within the day.

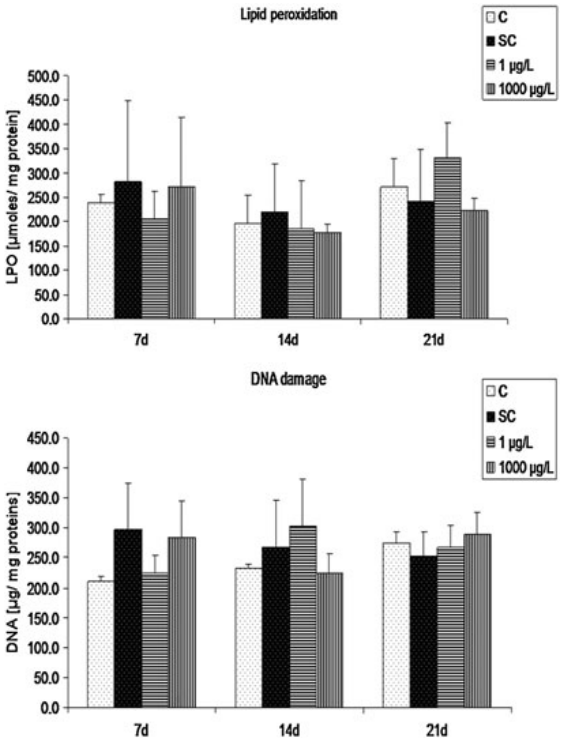

Figure 3. Expression of lipid peroxidation (LPO) and DNA damage (mean ± stdev) of blue mussels exposed to 1 μg/l and 1000 μg/l GEM. Significance set at $p \leq 0.05$.

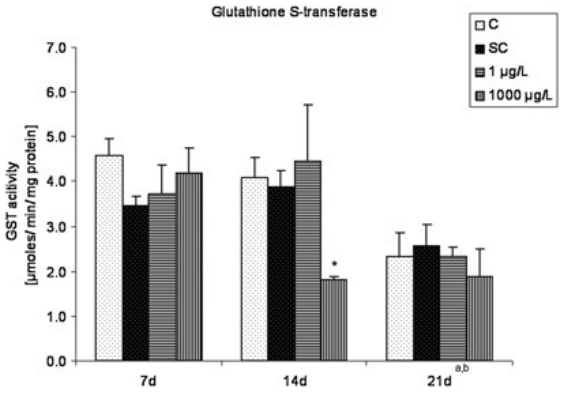

Figure 4. Expression of GST activity (mean ± stdev) of blue mussels exposed to 1 μg/l and 1000 μg/l diclofenac. Significance set at $p \leq 0.05$ with (a) indicating differences to day 7, (b) to day 14 and (*) significant to control within the day.

GAPDH (id: 0155) was significantly down-regulated at both concentrations by the 14-day diclofenac exposure. Aconitase 1 (eID: 0005) was also significantly down-regulated by diclofenac after 7 and 14 days of exposure. In addition it was significantly up-regulated at 1 μg/l GEM after 21 days. The class 1 ADH, beta subunit (id: 0169) showed a significant up-regulation at both concentrations

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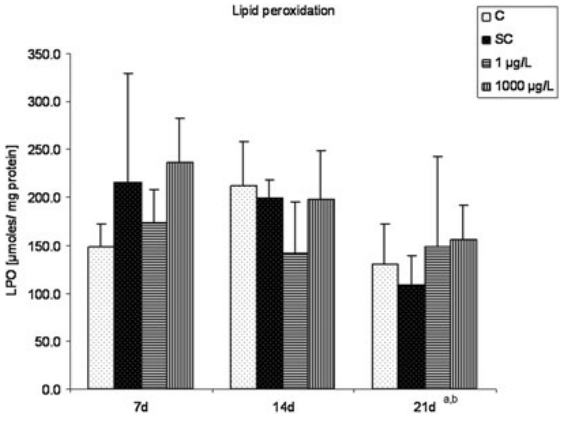

Figure 5. Expression of lipid peroxidation (LPO) (mean ± stdev) of blue mussels exposed to 1 µg/l and 1000 µg/l diclofenac. Significance set at $p \leq 0.05$ with (a) indicating differences to day 7 and (b) to day 14.

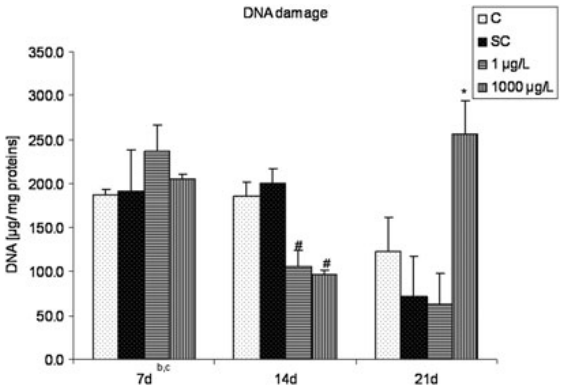

Figure 6. Expression of DNA damage (mean ± stdev) of blue mussels exposed to 1 µg/l and 1000 µg/l diclofenac. Significance set at $p \leq 0.05$ with (b) indicating differences to day 14, (c) to day 21 and (*) significant to control and (#) within the sampling day.

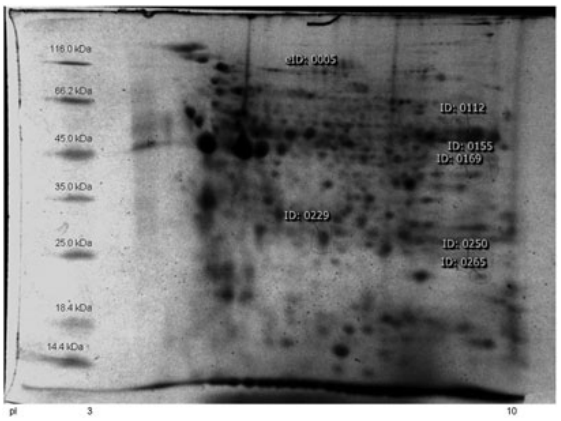

Figure 7. Annotated 2-DE image with the seven spots identified by LC-MS/MS (marked with their id number). The annotated spots were selected by combined PCA and ANOVA ($p \leq 0.05$ and with a fold change > 1.5).

Table 2. Differentially expressed proteins in the digestive gland of *Mytilus* spp. after the exposure to GEM and diclofenac for 7 and 14 days, as well as after the recovery week (21 days). Fold change of the up- and down-regulation is shown relative to control mussels

Protein Id	Protein	GEM 7 days		GEM 14 days		GEM 21 days		Diclofenac 7 days		Diclofenac 14 days		Diclofenac 21 days	
		1 µg/L	1000 µg/L	1 µg/L	1000 µg/L	1 µg/L	1000 µg/L	1 µg/L	1000 µg/L	1 µg/L	1000 µg/L	1 µg/L	1000 µg/L
eID:0004	?			+ 1.8	+ 1.4					- 1.5	- 1.2		
eID:0005	Aconitase 1, soluble					+ 1.7		- 1.1	- 1.4	- 1.7	- 1.4		
id:0223	?			+ 1.4	+ 1.3	+ 1.2	+ 1.7			+ 1.4	+ 1.5		
id:0169	Class 1 alcohol dehydrogenase, beta subunit					+ 2.2	+ 1.5		+ 1.1			+ 1.8	+ 2.0
id:0112	IDGF precursor					+ 2.7	+ 1.5					+ 1.8	+ 2.0
id:0288	?											- 1.2	- 1.3
id:0229	Hypothetical protein BRAFLDRAFT_282392							+ 1.3	+ 1.3	+ 1.7	+ 1.3	+ 1.5	+ 1.6
id:0155	GAPDH									- 1.2	- 1.6		
id:0265	Peptidyl-prolyl cis-trans isomerase 5 precursor										- 1.8		- 1.6
id:0250	Peptidyl prolyl cis-trans isomerase B					+ 2.1	+ 2.1						
id:0013	?			+ 2.3	+ 1.9								
id:0133	?			- 2.0									

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Table 3. Identified protein spots using LC-MS/MS

Spot ID	Protein	Acession number/ Mw (kDa)	Protein Score[a] / Protein Confidence Interval (%)	Total Ion Score/ Total Ion Confidence Interval (%)	Sequence Coverage (%)	Number of MS/MS patterns assigned to peptides[b]
eID:0005	Aconitase 1, soluble [*Xenopus (Silurana) tropicalis*]	gi|213982963/ 9.144	188/ 100	188/ 100	12	2
ID:0229	Hypothetical protein BRAFLDRAFT_282392 [*Branchiostoma floridae*]	gi|260831810/ 26.970	262/ 100	238/ 100	24	2
ID:0169	Class I alcohol dehydrogenase, beta subunit *[Xenopus (Silurana) tropicalis]*	gi|58332712/ 40.149	201/ 100	191/ 100	21	2
ID:0112	IDGF precursor [*Acyrthosiphon pisum*]	gi|274327724/ 48.622	93/ 99	86/ 100	16	1
ID:0155	GAPDH [*Plectospira myriandra*]	gi|34329029/34.115	144/ 100	121/ 100	18	1
ID:0265	Peptidyl-prolyl cis-trans isomerase 5 precursor [*Caligus clemensi*]	gi|225717940/ 17.942	446/ 100	390/ 100	42	5
ID:0250	Peptidyl prolyl cis-trans isomerase B [*Conus novaehollandiae*]	gi|289064185/ 22.600	138/ 100	103/ 100	53	1

[a]The protein score probability limit (where $p < 0.05$) is 85.
[b]Peptides with confidence interval above 95% were considered.

after 21 days pf GEM and diclofenac exposure. In addition it was significantly up-regulated after 7 days of diclofenac exposure. The two peptidyl prolyl isomerases (PPIase), peptidyl prolyl cis-trans isomerase B (id: 0250) and peptidyl-prolyl cis-trans isomerase 5 precursor (id: 0265), showed a difference in their expression during exposure to diclofenac and GEM. While peptidyl prolyl cis-trans isomerase B was significantly up-regulated at both concentrations of GEM after 21 days, peptidyl-prolyl cis-trans isomerase 5 precursor showed a significant down-regulation at 1000 μg/l after 14 and 21 days of diclofenac exposure. An imaginal disk growth factor (IDGF) precursor (id: 0112) showed a significant up-regulation after 21 days to both concentrations of both pharmaceuticals while a significant up-regulation by both concentrations over the entire exposure to diclofenac was seen for the hypothetical protein BRAFLDRAFT_282392 (id: 0229) (Figure 8).

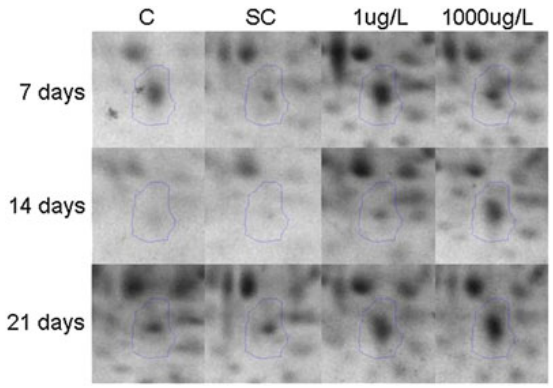

Figure 8. Representative 2-DE image of the spot (id: 0229) in the digestive gland of *Mytilus* spp. exposed to diclofenac. This spot was identified by LC-MS/MS as Hypothetical protein BRAFLDRAFT_282392 [*Branchiostoma floridae*].

Discussion

In the current study no negative effects of CF were observed and animals showed a generally healthy and unstressed behaviour, with their siphons extended indicating filtering. In addition CF values were in the same range as those previously published for wild animals taken from the same mussel population.[34]

Chemical analyses

Selection of suitable chemical concentrations for exposure is a crucial aspect of experimental design. In this study, a nominal environmentally relevant concentration (1 μg/l) was chosen as well as an elevated concentration of 1000 μg/l. Both concentrations of the pharmaceuticals have been previously shown to induce oxidative stress as well as damage in tissues of the blue mussel.[16]

Chemical analysis of the pharmaceuticals during the exposure showed the actual concentration of the nominal 1 μg/l concentrations at time 0 varied from 0.59–1 μg/l and 0.65–0.78 μg/l for GEM and diclofenac, respectively. This is within the range found in the aquatic environment.[6] Similarly, for the 1000 μg/l exposed mussels, the actual concentrations for both drugs at time 0 was around two-thirds lower than the nominal concentration. In addition it was observed that for the 1000 μg/l mussels exposed to both drugs (except diclofenac, 14 day), a higher actual concentration of the drug was found after 24 h than initially measured at time 0. This result was unexpected as most studies report a decrease in actual concentration over time, as was observed at

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the 1 μg/l exposure concentration, resulting from photodegradation, adsorption onto surfaces within the tank and absorption by the animal. A study by Cope *et al.*[35] showed that freshwater mussels of the superfamily *Unionoidae* successfully avoided exposure to the highest cadmium concentration tested by closing their valves. However this toxicant avoidance response was only maintained for the first 24 h of the exposure[35] and it can be assumed that such a response could be excluded in long-term exposures. Compounds with a log K_{ow} higher than or equal to three are considered to have the potential to bioaccumulate in biological tissue.[36] This is dependent on several other factors, however, such as uptake and depuration kinetics as well as different rates of metabolism in various organisms.[37] With a log K_{ow} of 4.77, it was assumed that GEM has the potential to bioaccumulate and or bioconcentrate in mussel tissue as has previously shown in goldfish.[15] In the current study, the possibility cannot be excluded that mussels may have reached their uptake capacity during the first week of exposure at this high concentration and, once saturated, subsequently may have begun to release the excess drug back into the water. This might, conceivably, provide a second route of exposure under high-dose conditions. Although mussels were fed before the water change at 24 h to try to reduce the influence of feeding on drug uptake, this may be a factor contributing to these results by offering a second route of exposure for the mussels. As there has been a general lack of data investigating the fate, uptake and effects of pharmaceuticals in aquatic organisms, this hypothesis is currently speculative.

Biomarker and proteomic responses

Exposure to GEM and diclofenac, respectively, induced oxidative stress in mussels, indicated by GST as an enzymatic biomarker of stress. A significant reduction of GST activity in mussels at day 21, previously exposed to diclofenac, was found. In addition, lower levels of LPO and DNA damage at day 21 suggest potential recovery from diclofenac exposure. These results confirm that diclofenac potentially induces oxidative stress and cellular damage at environmentally (1 μg/l) and elevated (1000 μg/l) concentrations as seen in a previous study.[16] In contrast to diclofenac exposure, mussels showed a higher GST level at day 21, after a 7-day recovery phase from GEM but no significant changes in the antioxidant system (LPO and DNA damage) were evident.

Environmental proteomics is a potentially robust and powerful tool for identification of mechanisms of interaction between toxicants and proteins.[38] Several studies have shown successful application of environmental proteomics after exposure to a variety of environmental contaminants (PCBs, PAH, heavy metal, and crude oil) using bivalve molluscs.[39] To our knowledge, this is the first proteomic study showing effects of the commonly found pharmaceuticals (diclofenac and GEM) over the course of a 14-day exposure, followed by a 7-day recovery phase.

Twelve spots were observed to be significantly changed by exposure to one and/ or both of the pharmaceuticals. Of these, seven proteins were successfully identified by LC-MS/MS and these belong to several protein families with different physiological functions. One of the proteins is GAPDH (id: 0155), an enzyme catalyzing the sixth step of glycolysis.[24] However, in addition to its role in glucose metabolism it carries out other functions in cellular processes.[24] Under oxidative stress a higher amount of the antioxidant cofactor NADPH is required, which is produced mainly by the pentose phosphate pathway.[40] Inactivation of GAPDH allows glucose metabolism to be diverted temporally to the pentose phosphate pathway, enabling the cell to generate more NADPH.[40] In the present study, GAPDH was significantly down-regulated at both concentrations of diclofenac after 14 days of exposure, suggesting oxidative stress due to the treatment with diclofenac. A hypothetical protein, BRAFLDRAFT_282392 (id: 0229), was significantly up-regulated at both concentrations of diclofenac exposure. This protein contains a domain (cd00311) that is linked to triose-phosphate isomerase (TIM), also a glycolytic enzyme, which catalyzes the interconversion of the triose phosphate isomerase dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (http://www.ncbi.nlm.nih.gov/gene/7248006). DHAP can be used to form fructose bis-phosphate, which is also required for the pentose phosphate pathway in order to activate NADPH formation. A further indicator of oxidative stress is the significant up-regulation of the class 1 ADH, beta subunit (id: 0169). The ADH protein superfamily catalyzes oxidation of various xenobiotics and endogenous alcohols to aldehydes. ADH1 is the classic liver enzyme responsible for ethanol metabolism[41] and has been shown to be induced by di(2-ethylhexyl) phthalate (DEHP) in *Chironomus*.[42] In addition, the Krebs cycle enzyme acotinase is a sensitive and specific marker for oxidative stress.[43] The identified aconitase 1 (eID: 0005) was significantly down-regulated during diclofenac exposure at 1000 μg/l at 14 and 21 days as well at 1 μg/l at 14 days. A decrease in its activity indicates oxidative stress due to elevated production of reactive oxygen species (ROS).[43] This has been previously shown to be also a sensitive marker for temperature and heavy metal stress in oysters.[43] These results suggest that mussels may still experience oxidative stress, even after termination of exposure.

While both peptidyl prolyl cis-trans isomerase 5 precursor (id: 0265) and peptidyl prolyl cis-trans isomerase B (id: 0250) belong to an enzyme family of folding catalysts, the PPIases,[44] they were differently expressed during exposures to diclofenac and GEM. PPIases are structurally conserved throughout evolution and commonly found in prokaryotes and eukaryotes.[44] With their chaperone-like activity they catalyze isomerization of proline peptide bonds from *cis* to *trans*, a process which is not only important for *de novo* protein folding, but also during the assembly of multidomain proteins.[44] In addition, PPIases show an affinity to the immunosuppressive drug cyclosporine A (CsA), which is widely used in prophylaxis and in treatment of allograft rejection following human organ transplants.[45] Furthermore, several cyclophilins, one of the PPIase family, have shown an extension of their role as molecular chaperones. Some of them have been identified as stress-inducible proteins which interact with heat shock proteins (hsps) in response to environmental stress, like thermal stress, ultraviolet irradiation and changes in the cell environment pH.[46] It is known that, in stressed cells, the level of unfolded, partially aggregated proteins increases and therefore also the need of hsps and other molecular chaperones in order to correct folding or prevent aggregation.[46] A significant induction of peptidyl prolyl cis-trans isomerase B after GEM exposure may indicate a role in repairing damage caused by protein misfolding. However, peptidyl prolyl cis-trans isomerase 5 precursor showed a different expression pattern, being down-regulated. It is possible that the peptidyl prolyl cis-trans isomerase 5 precursor has been modified to another form of the protein in response to 14 and 21 days of exposure to 1000 μg/l diclofenac, but this question will require further study. In the past, PPIase have been successfully identified in response to diseases and contamination (like PAHs) in bivalve molluscs which supports an important role for them in stress response.[47–49]

A proteomic evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine mussels (Mytilus spp.)

Drug Testing and Analysis

An IDGF, the IDGF precursor (id: 0112), was found to be significantly up-regulated after 21 days at both concentrations in both exposures of GEM and diclofenac. IDGFs have been previously identified and characterized in the Ecdysozoan model organism *Drosophila melanogaster*, which includes at least five family members and their characterization showed a high similarity to chitinase enzymes.[50] Furthermore it has been shown that IDGFs belong to a protein family which includes mammalian secreted glycoproteins of ill-defined function.[50] The human homolog HC gp-39 (16–23% identical with IDGFs) has been identified as a secretory product of articular chondrocytes and synovial cells from patients with arthritis.[51] It is also one of the antigens stimulating the autoimmune response in rheumatoid arthritis.[52] From day 14 to day 21 mussels were left without treatment in order to investigate their potential for recovery. Up-regulation of the IDGF precursor in *Mytilus* spp. at day 21 may possibly indicate an immune response to an inflammatory state triggered by both pharmaceuticals at both concentrations.

In conclusion, this study demonstrates use of an environmental proteomic approach in order to detect a unique PES for blue mussels in response to exposure to two commonly used human pharmaceuticals. This approach reveals potential toxicity mechanisms for both diclofenac and GEM in a non-target organism. Both drugs induced oxidative stress, involved molecular chaperones and seem to have had negative effects on the immune response of blue mussels. Oxidative stress was also observed at the enzymatic level as evidenced by GSTs. In addition, changes in the PES over time suggested that mussels were still partially experiencing oxidative stress for up to seven days post exposure.

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