

Title	Proteomic approaches to environmental stress in mussel <i>Mytilus edulis</i> due to emerging classes of anthropogenic pollutants
Authors	Jaafar, Siti NurTahirah
Publication date	2015
Original Citation	Jaafar, S. N. 2015. Proteomic approaches to environmental stress in mussel <i>Mytilus edulis</i> due to emerging classes of anthropogenic pollutants. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2024-04-18 13:13:37
Item downloaded from	https://hdl.handle.net/10468/2102

Proteomic Approaches to Environmental Stress in Mussel *Mytilus edulis* Due to Emerging Classes of Anthropogenic Pollutants

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SCHOOL OF BIOCHEMISTRY AND CELL BIOLOGY

Thesis submitted for the degree of

Doctor of Philosophy

April 2015

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Declaration

I hereby declare that this thesis presented for the degree of Doctor of Philosophy to the National University of Ireland has not previously been presented for a higher degree to this or to any other university. Any assistance provided is acknowledged in the text by reference and to the researchers or their publications. This thesis is of my own composition and may be photocopied or lent to other libraries for the purpose of consultant.

Siti NurTahirah Jaafar

Acknowledgement

I would like to express my deepest appreciation to my supervisor Professor David Sheehan who has always guide, conveyed positive environment and opportunity towards my research. Without his guidance and persistent help this thesis would not have been possible. I would like to thanks Ministry of Education Malaysia and University Malaysia Terengganu for the opportunity and financial support.

A million thanks to Louis-Charles Rainville, Wentao Hu and Sara Tedesco. You always help me out with my methodology and laboratory skills. I can't thank you enough for everything that you have done for me. Not forgettable, Noreen, Pat, Trish and Jenny, who always there when I needed help.

Last but not least, a huge appreciation to my family and friends especially my husband (Abdul Ghani) and my children (Afia and Ain) who always understand and supporting me all these years. For my parents (Abah and Umami), siblings, my big family (aunties and uncles) and in-laws family, thank you for always pray for my success. Not forgettable, my thought and infinite love to my late grandmother, who know and believed me more than myself.

Terima kasih semua!

‘**T**uhan jangan kau izin aku lupa,
Jangan kau biarkan aku mendepang dada,
Pasakkan hatiku pada kaki,
Paksakan ku tunduk sehingga mati’

Fynn Jamal

Abstract

Anthropogenic pollutant chemicals pose a major threat to aquatic organisms. There is a need for more research on emerging categories of environmental chemicals such as nanomaterials, endocrine disruptors and pharmaceuticals. Proteomics offers options and advantages for early warning of alterations in environmental quality by detecting sub-lethal changes in sentinel species such as the mussel, *Mytilus edulis*. This thesis aimed to compare the potential of traditional biomarkers (such as enzyme activity measurement) and newer redox proteomic approaches. Environmental proteomics, especially a redox proteomics toolbox, may be a novel way to study pollutant effects on organisms which can also yield information on risks to human health. In particular, it can probe subtle biochemical changes at sub-lethal concentrations and thus offer novel insights to toxicity mechanisms. In the first instance, the present research involved a field-study in three stations in Cork Harbour, Ireland (Haulbowline, Ringaskiddy and Douglas) compared to an out-harbour control site in Bantry Bay, Ireland. Then, further research was carried out to detect effects of anthropogenic pollution on selected chemicals. Diclofenac is an example of veterinary and human pharmaceuticals, an emerging category of chemical pollutants, with potential to cause serious toxicity to non-target organisms. A second chemical used for this study was copper which is a key source of contamination in marine ecosystems. Thirdly, bisphenol A is a major anthropogenic chemical mainly used in polycarbonate plastics manufacturing that is widespread in the environment. It is also suspected to be an endocrine disruptor. Effects on the gill, the principal feeding organ of mussels, were investigated in particular. Effects on digestive gland were also investigated to compare different outcomes from each tissue. Across the

three anthropogenic chemicals studied (diclofenac, copper and bisphenol A), only diclofenac exposure did not show any significant difference towards glutathione transferase (GST) responses. Meanwhile, copper and bisphenol A significantly increased GST in gill. Glutathione reductase (GR) enzyme analysis revealed that all three chemicals have significant responses in gill. Catalase activity showed significant differences in digestive gland exposed to diclofenac and gills exposed to bisphenol A. This study focused then on application of redox proteomics; the study of the oxidative modification of proteins, to *M. edulis*. Thiol proteins were labelled with 5-iodoacetamidofluorescein prior to one-dimensional and two-dimensional electrophoresis. This clearly revealed some similarities on a portion of the redox proteome across chemical exposures indicating where toxicity mechanism may be common and where effects are unique to a single treatment. This thesis documents that proteomics is a robust tool to provide valuable insights into possible mechanisms of toxicity of anthropogenic contaminants in *M. edulis*. It is concluded that future research should focus on gill tissue, on protein thiols and on key individual proteins discovered in this study such as calreticulin and arginine kinase which have not previously been considered as biomarkers in aquatic toxicology prior to this study.

Abbreviations

1DE	: One-dimensional electrophoresis
2DE	: Two-dimensional electrophoresis
ANOVA	: Analysis of variance
Arg	: Arginine
ASW	: Artificial sea water
ATP	: Adenosine triphosphate
ATS	: Activated thiol sepharose
BPA	: Bisphenol A
BSA	: Bovine serum albumin
CAT	: Catalase
CDNB	: 1-chloro-2,4-dinitrobenzene
CHAPS	: 3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
FTSC	: Fluorescein-5-thiosemicarbazide
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
GR	: Glutathione reductase
GSH	: Reduced glutathione
GST	: Glutathione transferase
GSSG	: oxidised glutathione
HP	: Hypothetical protein
HSC	: Heat shock cognate protein
HSP	: Heat-shock protein
IAF	: 5-iodoacetamidofluorescein
IPG	: Immobilised pH gradient
MALDI	: Matrix assisted laser desorption/ionisation

MS	: Mass spectrometry
NADPH	: Nicotinamide adenine dinucleotide phosphate
NCBI	: National center for biotechnology information
NR	: Neutral red
NRRT	: Neutral red retention time
OMPs	: Outer membrane proteins
OS	: Oxidative stress
PES	: Protein expression signature
PMSF	: Phenylmethanesulfonyl fluoride
PTM	: Post-translational modifications
ROS	: Reactive oxygen species
SOD	: Superoxide dismutase
SDS-PAGE	: Sodium dodecylsulfate polyacrylamide gel electrophoresis
TCA	: Trichloroacetic acid

CHAPTER 1: INTRODUCTION

1.1 General introduction

Environmental toxicology is an aspect of the fields of environmental science and environmental studies (Wright and Welbourn, 2002). It explores how exposure to toxic chemicals (especially of human or “anthropogenic” origin) can potentially alter the health of living organisms (fish, wildlife, humans) and impact on the quality of the natural environment with possible long-term implications for human health (Younglai et al. 2007). Figure 1 is a schematic illustration of some key components of environmental toxicology. The elements highlighted in this scheme provide information that can potentially be used in risk assessment (Wright and Welbourn, 2002). This information can help us in assessing the potential risks of chemical substances to humans, animals or other aspects of environmental health.

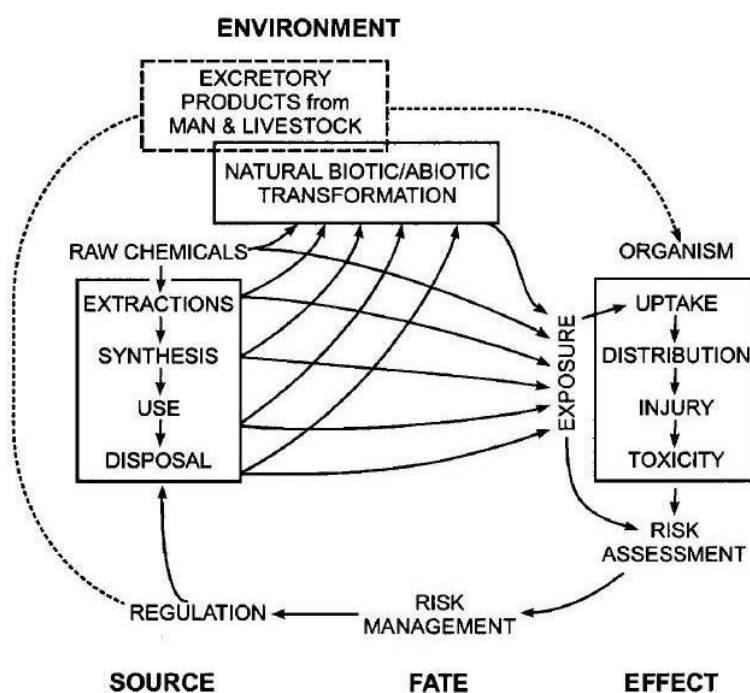


Figure 1. Principal components of environmental toxicology (Wright and Welbourn, 2002)

Chemicals become distributed within the environment through transport processes in water, air, and soil. These transfer media consist of solid, liquid or gaseous phases and transfer processes can be especially challenging to model to determine the ultimate environmental fate of individual chemicals. Often, specific chemicals end up in reservoirs such as environmental sinks (e.g. sediments) where they may pose a long-term pollution risk. The movement of chemicals through a transporter medium may significantly impact living organisms, the biota, both as individuals, as populations and as ecological communities (Kenaga, 1980). Moreover, a variety of environmental processes may alter the chemical structure of pollutants. For example, photooxidation can oxidise chemicals over time which gives rise to speciation in the case of metal pollutants, crude oil and polycyclic aromatic hydrocarbons (Bobinger and Andersson, 2009). Similarly, there are environmental processes which challenge organisms in a particular location. For example, organisms at the edge of their natural range can be challenged by global warming processes (Tomanek, 2012; Applebaum et al., 2014; Morris et al., 2015), by ocean acidification (Doney et al., 2012) and by competition from invasive species (Torchin and Mitchell, 2004). These may alter the community of species in a particular location and can influence the ability of a specific species to withstand further challenges posed by anthropogenic pollutants. There is therefore a very complex three-way relationship between environment, chemicals and biota as summarised in Figure 2.

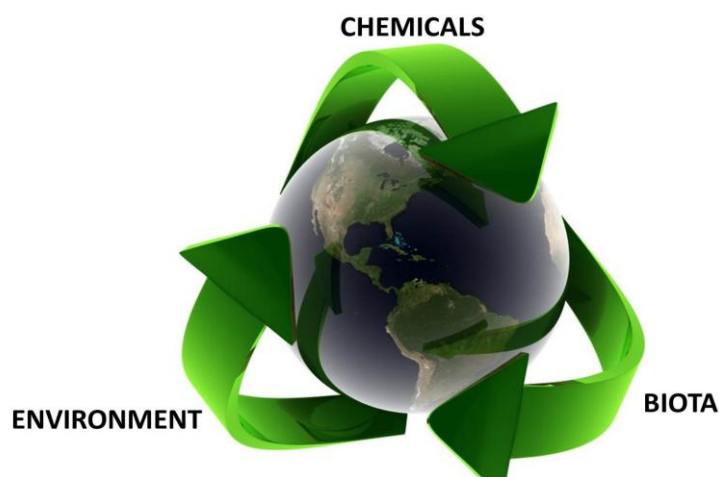


Figure 2. Relationship between environment, chemicals and biota.

The multiple routes through which pollution can access the natural environment, especially the sea, could potentially produce an unsafe environment for marine organisms. This would have the effect of degrading the environmental quality of coastlines and estuaries with consequent effects on biodiversity, flood-defences, fish/shellfish stocks and loss of valuable environmental services provided by these ecosystems (Borja et al. 2010). This potentially compromises the ability of the natural environment to cope with further stressors such as climate change and overpopulation. Much research has been carried out to investigate the effects of chemicals on marine organisms. The common marine species used as animal models are often benthic organisms, especially bivalves (Box et al. 2007). Biomarkers within sentinel organisms have been extensively used as a popular means to evaluate changes due to environmental pollution (Frenzilli et al. 2004). These comprise physiological, histological or biochemical indicators that suggest an organism has been exposed to xenobiotics. Common examples of biomarkers widely used in

ecotoxicology include the presence of imposex (penis or *vas deferens* appearing in female animals), eggshell thinning, lipid peroxidation and levels of glutathione transferases (Fitzpatrick et al., 1997; Forbes et al., 2005).

1.2 Ecotoxicology

Ecotoxicology is a term that combines ecology and toxicology and focuses on processes by which chemicals (pollutants) entering the natural environment (particularly anthropogenic chemicals) can exert toxic effects. In general, ecotoxicology involves a mixture of pollutants such as organic matter, trace metals and pesticides combined with a variety of environmental matrices (e.g. water, sediment, tissue) (Chapman, 1995). Anthropogenic toxic substances become environmental contaminants when they leave their target sites or move from safe to unsafe situations (Anderson, 1995). Figure 3 shows the various levels of toxicity studies in the environment based on diverse sources and timescale.

A key aim of ecotoxicology is to understand the mechanism of contamination and the pollutants' mode of biological action so as to develop suitable solutions to prevent adverse outcomes resulting from environmental contaminants. There is a variety of contaminant effects that can alter the ecological stability of organisms or populations (Connon et al. 2012). The ecotoxicology field therefore attracts interest from a wide variety of scientific disciplines ranging from ecologists to biologists and often including biochemists and analytical chemists. This has contributed to greater understanding of different anthropogenic chemicals capable of affecting species, interactions, communities and ecosystems (Relyea, 2006).

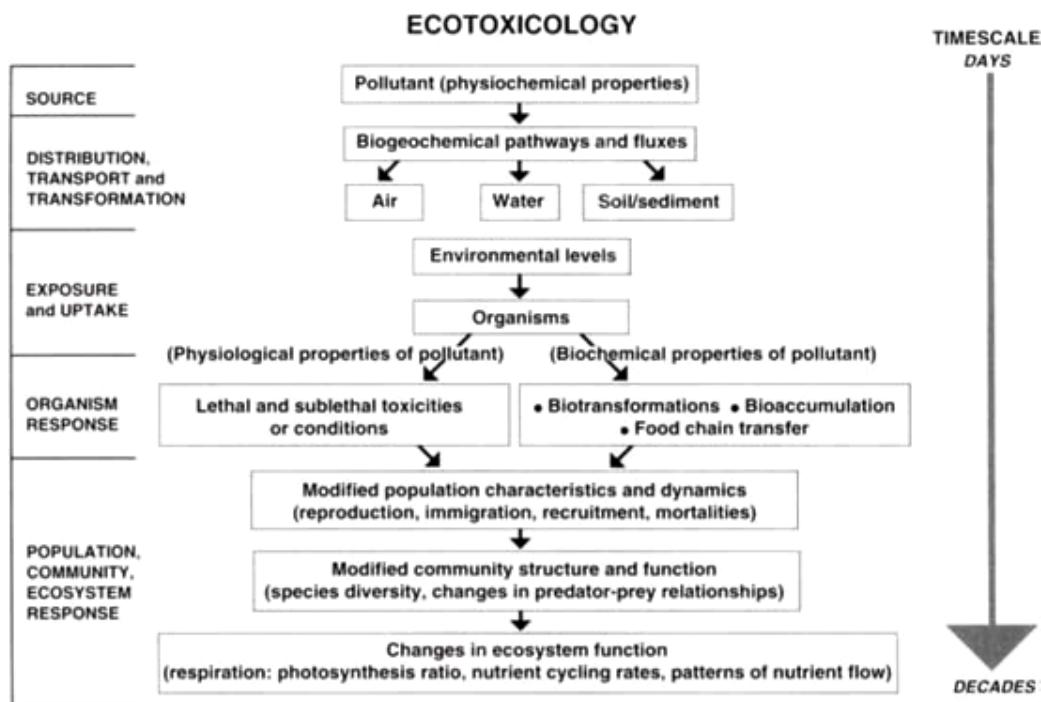


Figure 3. Various levels of toxicity studies in the natural environment. The diagram is summarized from many sources combining ecology and toxicology; ecotoxicology (Anderson, 1995).

A robust methodology is crucial to allow meaningful hazard assessment of toxicants in contact with aquatic organisms (Luigi, 2007). Early studies focused on specific biomarkers such as imposex (Losso and Ghirardini, 2010) and levels of acetylcholine esterase activity (Song et al., 1997). However, in more recent studies a methodology paradigm involving multivariate information has gradually replaced traditional single-parameter biomarkers. These often incorporate traditional biomarkers along with measures taken to reflect biochemical changes in response to pollutants. Omics technologies have found particular popularity in this regard since complements of key biomacromolecules such as RNA and proteins would be expected to alter in response to profound chemical challenge facilitating insights to

biochemical mechanisms of toxicity and discovery of biomarkers not predicted *a priori* (Dowling and Sheehan, 2006; Ge et al., 2013). Robust molecular biomarker measurements of protein expression or gene levels would be expected to allow early detection of environmental stress upon exposure to chemicals or stressor (Luigi, 2007; Dowling and Sheehan, 2006; Ge et al., 2013). These approaches are discussed in more detail below (Section 1.6).

1.3 Anthropogenic pollutants

Anthropogenic pollution has existed since human beings first started using fire and smelting metals. Nonetheless, as the world has become ever more industrialised, new man-made compounds have contributed to an increasingly broad range of contaminants (Hong et al. 1996). A majority of the globe's human population live within 100 km of coastlines and most large urban centres are located on or near marine estuaries (Borja, 2005). It is thought that there are in excess of 100,000 anthropogenic materials in the environment arising from industrial, shipping, building and other human activities and many of these act as chemical pollutants on coastlines and estuaries which often function as sinks for such pollutants (Borja et al. 2010). The sheer range and complexity of anthropogenic chemicals present sometimes in only trace amounts in the environment exceeds the capacity of analytical chemistry on its own which has led to surveillance approaches using sentinel species as a complement to direct chemical analysis (Goldberg et al., 1978).

Anthropogenic pollution disturbs natural ecosystems and their damaging effects are not considered to have ceased until secondary succession (e.g. flood, harvesting, and forest fire) has returned the ecosystem to its pre-existing state. This recovery action involves a scale of time, space and intensity of anthropogenic

disturbance (Borja, 2010). Scientists have become extensively concerned about possible adverse health effects on humans and wildlife because of environmental exposure to industrial, pharmaceutical, and natural chemicals with estrogenic, androgenic, or thyroid-disrupting properties (Benninghoff, 2006). The biological effects of anthropogenic pollutants diverge substantially between organisms reflecting differences in modes of exposure, routes of uptake, metabolism following uptake, rates of accumulation and sensitivity of the target organs and differences in excretion (Rhind, 2009). In recent years, a number of new classes of environmental pollutants have been identified which are regarded as emerging categories of anthropogenic pollutants (Clarke and Cummins, 2015; Gavrilescu et al., 2015). These include: endocrine disruptors (Quinn et al., 2004; Crain et al. 2007; Gavrilescu et al., 2015), nanomaterials (Tedesco et al. 2010) and pharmaceuticals intended for human or animal targets (Quinn et al., 2008; Zhang et al. 2008; Clarke and Cummins, 2015; Gavrilescu et al., 2015). This thesis focuses on examples of two of these three categories of chemicals (benchmarked with a more traditional pro-oxidant pollutant; Cu Cl_2) and exploits redox proteomics methods to explore their consequences for the bivalve *Mytilus edulis*.

1.3.1 Pharmaceuticals; diclofenac

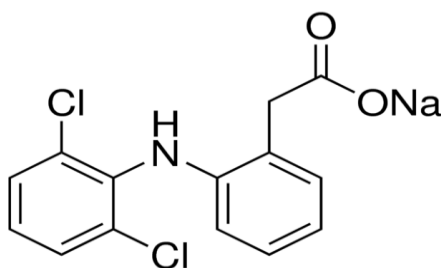


Figure 4. Chemical structure of diclofenac.

Pharmaceutically active compounds (PhACs) in the aquatic environment have been recognized as one of the emerging categories of anthropogenic pollutants in ecotoxicology (Heberer, 2002; Quinn et al., 2008; Clarke and Cummins, 2015; Gavrilescu et al., 2015). Pharmaceuticals are designed to cause potent biological effects at low doses in human and animal targets and are often formulated to be long-lasting and persistent within the body. They therefore can cause unintended effects on non-target aquatic organisms and this has been investigated in acute toxicity assays. Low levels of human medicines (pharmaceuticals) have been detected in many countries in sewage treatment plant (STP) effluents, surface waters, seawaters, groundwater and some drinking waters (Fent et al. 2006; Quinn et al., 2008). Diclofenac (Figure 4) is a well-known non-steroidal anti-inflammatory drug (NSAID) that is widely used to relieve acute pain and symptoms of rheumatoid arthritis. The drug works as a cyclooxygenase 2 inhibitor and the mechanism of interaction with this target has been elucidated by structural biology (Rowlinson et al., 2007). Cyclooxygenase 2 plays a key role in conversion of arachidonic acid into prostaglandin H₂ in the biochemical pathway by which polyunsaturated fatty acids are converted to prostaglandins and thromboxanes (Mardini and Fitzgerald, 2001). Cyclooxygenase 2 is especially important in inflammatory cells and the central nervous system so cyclooxygenase 2 inhibitors reduce inflammation. Diclofenac is one of the most popular PhACs in use in the general population.

However, diclofenac has also been reported as causing a decline in the population of the Indian vulture *Gyps sp* due to its known toxicity to kidneys and to induce extensive oxidative stress in brain, liver and blood of the common carp *Cyprinus carpio* (Oaks et al. 2004, Islas-Flores et al. 2013). This led to ecological effects such

as an increase in feral dogs in India taking advantage of the new ecological niche opened up by the vulture's disappearance. Interestingly, the turkey vulture, *Cathartes aura*, can withstand a one hundred times higher dose of diclofenac than *Gyps sp* illustrating how species-specific such toxicity can be (Rattner et al., 2009). Because of concerns about its toxicity to non-target species (Cleuvers, 2001; Fent et al., 2006), diclofenac (along with 17 α -ethinylestradiol and 17 β -estradiol) was added in 2013 to a “watch” list of priority substance (suspected toxic environmental chemicals) under the European Union's Water Framework directive (http://ec.europa.eu/environment/water/water-dangersub/pri_substances.htm).

In the bivalve mollusc, *Mytilus galloprovincialis* diclofenac has been reported to rapidly stimulate the antioxidant system of gill tissue and some damage was confirmed in digestive gland after a two-week exposure (Gonzales-Rey and Bebianno 2014). The “lowest-observed effect” levels of diclofenac for liver, kidney and gill of rainbow trout is approximately 1 $\mu\text{g L}^{-1}$ (Triebskorn et al., 2007). This dosage also caused extensive cell and gene toxicity in the Japanese medaka fish, *Oryzias latipes* (Hong et al., 2007). A genotoxicity study has also recently shown that, while diclofenac is very toxic to the soil arthropod *Folsomia candida*, there is some uncertainty about its comparative mode of action in differing organisms (Chen et al., 2015).

1.3.2 Heavy metals; Copper (II) Chloride

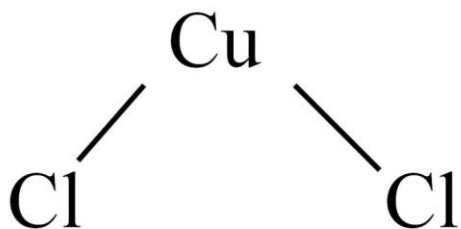


Figure 5. Chemical structure of Copper II chloride

Copper is one of the most ubiquitous contaminants found in many industrial, nonpoint source (NPS) effluents such as, mining, smelting, farmland and also as natural elements (Flemming and Trevors, 1989). It is abundant in the Earth's crust and occurs at low levels in seawater (Flemming and Trevors, 1989; Arnold et al., 2009). Copper occurs as ionisable salts such as CuCl_2 (Figure 5) and as insoluble or sparingly soluble copper compounds such as metal ores and the minerals chalcopyrite, chalcocite and bornite (Majuste et al., 2013).

Copper performs many key biochemical functions as it is an essential micronutrient. However, at high concentrations, copper is also toxic so it has a U-shaped dose-response curve (Stern et al., 2007). Copper deficiency was first discovered by Hart et al. (1928) in rats fed a copper-free milk diet that went on to develop anaemia. It is a component of metalloenzymes such as superoxide dismutases, laccases and oxidases where it is involved in accepting or donating electrons by cycling between the Cu I and Cu II oxidation states (Stern et al., 2007; Komori et al., 2014). It also frequently contributes to maintenance of protein structure and in DNA binding through its coordination chemistry (Sea et al., 2015).

The most toxic forms of copper in the environment are thought to be ionisable salts such as CuCl_2 . The principal route of ingestion in most organisms is oral and copper is consistently one of the most toxic dietary metals, especially to aquatic organisms (Arnold et al., 2009; DeForest and Meyer, 2015). Importantly, copper's toxicity to biota is heavily influenced by factors such as the presence of dissolved carbon (Gillis et al., 2009; Giacomini et al., 2013) and pH (Lofts et al., 2013; Ivanina and Sokolova, 2013). In the environment, binding to material such as dissolved carbon significantly reduces copper toxicity (Giacomini et al., 2013). Geologically-occurring copper may be concentrated by human activity such as mining and smelting and subsequently deposited in the marine environment (Seligman, 1998; Rhind, 2009). Copper levels released from chromated copper arsenate-treated wood submerged in seawater are high initially but decrease with time and this treatment process is known to be toxic to non-target marine organisms (Vanbroek and Resli, 1999).

1.3.3 Endocrine disruption, Bisphenol A

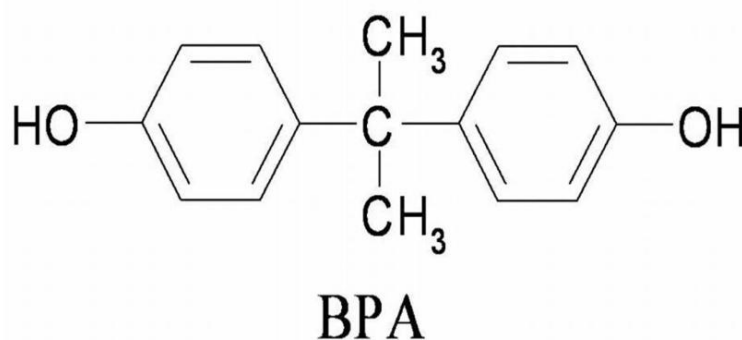


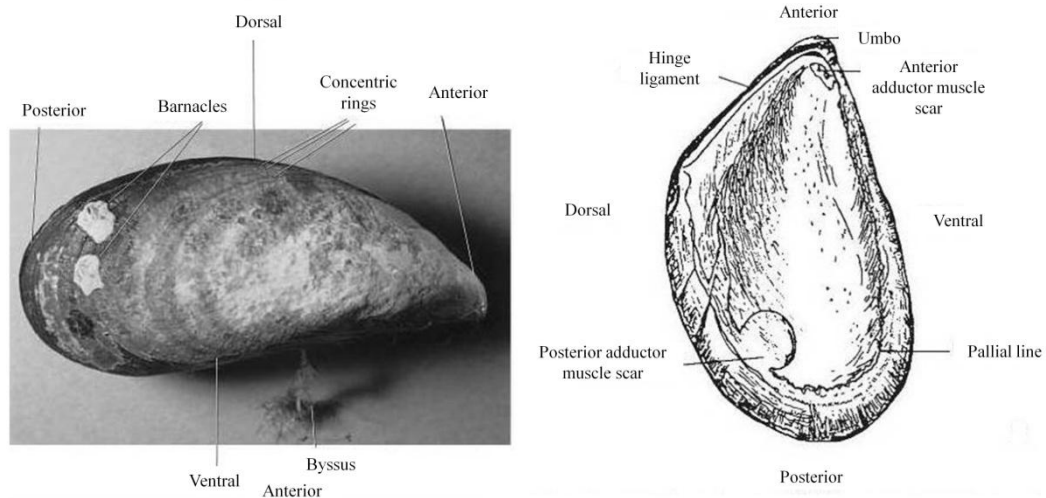
Figure 6. Chemical structure of Bisphenol A (Kang, 2007).

Endocrine-disrupting chemicals represent an important emerging category of anthropogenic chemicals commonly found in the environment. These are defined as exogenous chemicals or chemical mixtures that impact on endocrine system structure or function and cause adverse effects (Flint, 2012). Endocrine disruption refers to an intrusion into endocrine system function by environmental chemicals (Witorsch, 2002) and this effect has been reported for mussels (*Dreissena polymorpha*) exposed to municipal effluent (Quinn et al., 2004). More than 66 chemicals have been identified by the European Commission as potentially posing endocrine disrupting risks to humans including chlordane, nonylphenol, polychlorinated biphenyls, tributyltins, aroclors, resorcinol and bisphenol A [BPA; 2, 2-bis (4-hydroxyphenyl) propane, 4,4'-isopropylidenediphenol] (Figure 6) (EC, 2015).

BPA has long been suspected as an endocrine disruptor and is known to be acutely toxic to aquatic organisms in the range of 1000–10,000µg/L for freshwater and marine species (Alexander et al. 1988; Goodman et al. 2006). This material is extensively used in the manufacture of polycarbonate plastics and epoxy resins. Approximately 770 billion grams of BPA is synthesized in the US *per annum* and it is one of the highest-volume chemical products produced worldwide (Crain et al. 2007). There are concerns about BPA's toxicity, especially during neurodevelopment of children, puberty and pregnancy. The material is now banned from the manufacture of babies' drinking bottles in Canada and Denmark. Because of these concerns about BPA's potential as an endocrine disruptor, the US National Institute of Environmental Sciences has launched several research initiatives to bring basic scientists together with scientists working in accredited laboratories favoured by the six US companies that produce BPA commercially (https://www.niehs.nih.gov/research/programs/endocrine/bpa_initiatives/index.cfm).

There have also been important international reports from supranational bodies such as the European Food Safety Authority and the UN's Food and Agricultural Organisation/World Health Organisation discounting toxicity at current recommended doses (EFSA, 2010; FAO/WHO, 2010). Interestingly from the perspective of aquatic toxicology, a well-cited review has concluded that, while laboratory studies point to endocrine disruptors being toxic to many fish species in laboratory studies, this has not affected reproduction of several fish species in the field (Mills and Chichester, 2005).

1.4 The bivalve, *Mytilus edulis*; An animal model in ecotoxicology



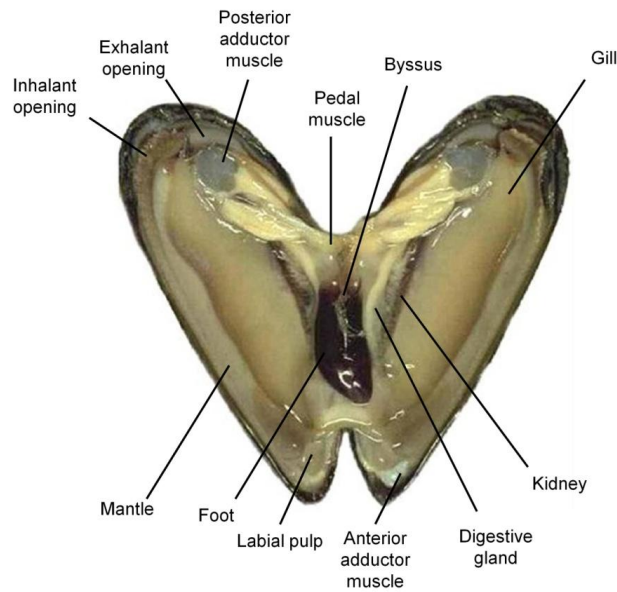


Figure 7. External and internal view of *Mytilus edulis* (modified from Gosling, 2008)

Mussels are the category of marine species most commonly used for marine pollution monitoring. This is because they are sessile organisms with high filtration rates, easy recognition and handling, and wide geographical distribution which are nonetheless reasonably robust to most forms of chemical pollution (Widdows and Donkin, 1992). Figure 7 shows images of *Mytilus edulis* (modified from Gosling, 2008). *M. edulis* is a filter-feeding bivalve which filters large volumes of water daily for respiratory and feeding purposes. They can accumulate many pollutants in their tissues which is sometimes helpful in chemical analysis, for example with metals (Sokolova et al. 2005). High to moderate pollutant concentrations are sometimes more easily detected within the tissues of this animal compared to direct measurements in other environmental samples such as sediment and water (Sericano, 2000; Crowe et al. 2004). Mussels also provide important ecological services in estuaries (where much human habitation is located) and are key food-sources in

some regions of the World (e.g. Spain, Italy, China) where aquaculture is particularly well-developed (FAO, 2014).

1.4.1 Use of *Mytilus spp* in ecotoxicology

Mytilus spp is the most popular marine bivalve genus used worldwide as a sentinel in ecotoxicology investigations of contamination with anthropogenic chemicals (O'Connor 2002). It has been used in large-scale screening programmes, for example, the Mussel Watch Program of the US National Oceanic and Atmospheric Administration (NOAA) in the years between 1986 and 2005 (Goldberg et al., 1978; Goldberg and Bertine, 2000). This programme showed that mussels can accumulate contaminants which are typically found near urban and industrial areas of estuarine and coastal systems (Kimbrough et al. 2008). Mussels have a very broad geographical distribution and differing varieties and hybrids are found in most temperate regions of the World (Gosling, 2008). They are easy to identify, to collect and abundant in estuarine waters. They can accumulate high levels of trace metals and organic compounds within their tissues, particularly gill and digestive gland (Lau and Wong, 2003). Because of this, mussels can provide a time-integrated indication of environmental contamination with observable cellular and physiological responses. These responses make them exemplary sentinels for environmental monitoring (Goldberg and Bertine, 2000; Manduzio et al., 2004). Mussels are euryhaline and widespread in coastal environments and thus contribute significant ecological services such as habitats for various combinations of invertebrates and algae, a food-source for migrating birds and water-filtration in

estuaries. In contrast to some other marine organisms, mussels are well-adapted to coping with relatively high levels of many pollutants (Crowe et al. 2004).

1.4.2 Chemical uptake by *Mytilus spp*

Observable monitoring and biomarker measurement of pollutant impact on mussels is influenced by understanding of cellular physiological and biochemical processes (Roseijadi et al, 1994 and Ballatori, 2002). For instance, nonessential metals (e.g. mercury, lead and cadmium) are frequently taken up by the same uptake pathways and membrane carriers that have evolved for essential metals (Devoid and Etter, 2007). When absorption processes occur, these types of metals are transported *via* the bivalve's open circulatory system to various internal tissues, where accumulation, utilization and, in some cases, toxicity can occur. Much research has been carried out to investigate mechanisms of metal uptake, intracellular regulation, and detoxification in marine bivalves (Cosson, 2000; Rainbow, 2000). Controlled exposure of marine and freshwater mussels has allowed profiling of endocrine disruptors in mussel tissues and determination of toxicity and bioconcentration factors (Quinn et al., 2006; Gatidou et al., 2010). Interestingly, the bioconcentration factor for BPA was more than twice that for nonylphenol in *M. galloprovincialis* (Gatidou et al., 2010).

On the other hand, research into environmental effects of pharmaceuticals is relatively scarce (Gagne et al., 2006; Quinn et al., 2008; Arnold et al., 2014). The concentrations of individual pharmaceuticals in aquatic wildlife are at least 1 order of magnitude higher than those measured in surface waters. They are generally detected in wastewater effluents and surface waters in the nanogram to microgram

per liter concentration range. Research has demonstrated that analgesics caused cellular oxidative damage only at concentrations 10-100 times higher than those detected in waste water effluents (Gagné et al., 2006).

1.5 Oxidative stress (OS) and reactive oxygen species (ROS)

Aerobic metabolism is fifteen times more efficient at producing ATP than anaerobic metabolism. More than two billion years ago, biology on Earth shifted from predominantly anaerobic to predominantly aerobic energy metabolism and this, eventually, enabled cells to increase greatly in size, to form multicellular organisms and new phyla. This *Great Oxygenation Event* changed Earth's atmosphere from a reducing to an oxidising one (Lalonde and Konhauser, 2015). However, oxygen is potentially toxic to cells as production of ROS during reduction of O₂ in mitochondrial electron transport chains is strongly thermodynamically favoured (Davies, 2005). Therefore, cells have needed to adapt to the presence of high levels of ROS by development of extensive antioxidant systems including small antioxidant molecules like reduced glutathione (GSH), antioxidant enzymes like catalase and other proteins acting as redox buffers (Winterbourn, 2008; Sheehan et al., 2010). It should also be noted that much of the cell's antioxidant defences require NADPH + H⁺ reducing equivalents (rather than NADH + H) so, in response to a redox challenge, there is a need to divert glucose from the usual glycolytic pathway to the pentose phosphate pathway (Tomanek, 2012). This is an aspect of cells and organisms rapidly adapting their metabolism to cope with toxic challenges (Tomanek, 2012; Applebaum et al., 2014).

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential cellular damage (Sies, 1997; Kelly, 2003). This can arise as a result of endogenous antioxidant defences being impaired and/or overwhelmed due to over-high levels of ROS (Fig. 8) (Kirkham and Barnes, 2013). Chemical species such as oxygen free radicals and other ROS have potential to damage tissues and crucial cellular components including proteins, DNA, small molecules and membrane lipids (Davies, 2005; Winterbourn, 2008). Many ROS react with biological targets with extremely high rate constants, in some cases limited only by the rate of diffusion (Davies, 2005).

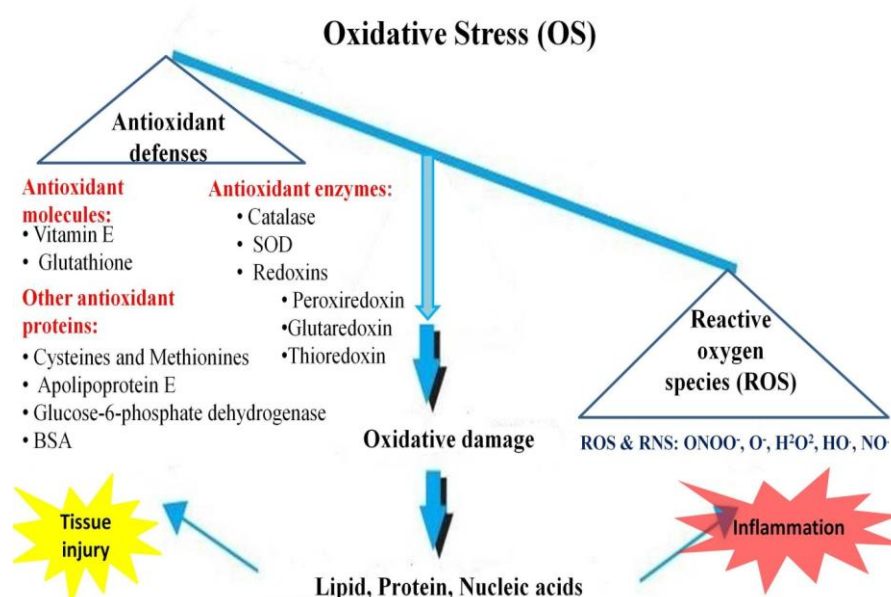


Figure 8. Oxidative stress occurs when there is an excess of ROS over antioxidant defences. ROS concentrations exceeding available antioxidant defences may oxidise cell components (Modified from Kelly, 2003; Sheehan et al. 2012).

This is increasingly relevant to ecotoxicology because oxidative damage is often induced by many classes of chemical pollutants (Livingstone, 2001; Valavanidis et al. 2006). Moreover, ROS have been shown to be involved in

oxidative cell damage in a range of processes such as apoptosis (Harrison et al 2005), ageing (Finkel et al 2000, Cui et al., 2012), nanoparticle (Tedesco et al, 2010) and pharmaceutical exposure (Schmidt et al 2014) (Fig. 9).

Marine animals are readily exposed to anthropogenic pollutants present in the water column, sediments or in prey items. Some individuals may be unhealthy, experiencing other stressors (e.g. climate change, competition from invasive species) or may be old all of which can compromise the animal's antioxidant defence systems (Livingstone, 2001; Finkel et al 2000, Cui et al., 2012; Sheehan et al., 2012). Amongst marine animals, bivalves are especially exposed to relatively high levels of pro-oxidants as a consequence of their filter-feeding habit.

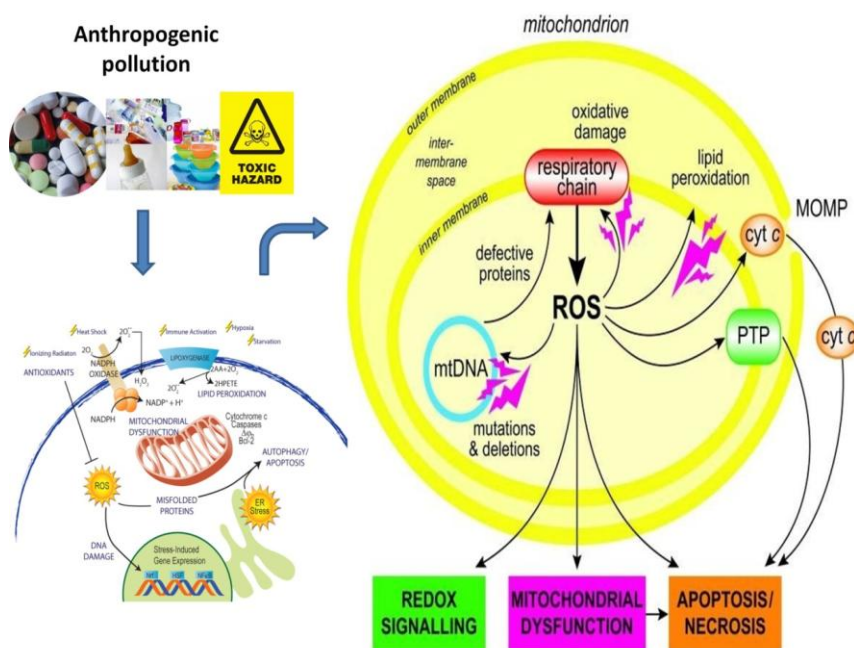


Figure 9. ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA. Modified from Murphy (2009).

1.6 Proteomics

The term *proteome* was originally coined by an Australian scientist, Mark Wilkins, in 1997 to denote the complement of proteins expressed in a biological preparation under a given set of experimental conditions (Wilkins et al., 1996). *Proteomics* represents a set of methodologies to perform large-scale analysis of complex protein mixtures, notably using two-dimensional electrophoresis (2DE) (Wilkins et al., 1996) and/or mass spectrometry (MS) techniques (Aebersold and Mann, 2003). These terms are analogous to *genome* and *genomics* (DNA) and *transcriptome* and *transcriptomics* (RNA) and, taken collectively, they represent key parts of a systems biology approach to biological questions which is based on high-throughput methodologies combined with rapid data sorting and analysis including modelling (Ideker et al., 2001).

A big difference between *omics* approaches and more traditional biochemical studies is that the former are largely discovery-based rather than being driven by any particular hypothesis. In the context of ecotoxicology, it should therefore be possible to discover new biomarkers by this approach additional to traditional biomarkers mentioned in Section 1.2 above. The groups of Bradley and Tedengren pioneered this approach in *M. edulis* exposed to multiple stressors by exploiting 2DE (Shephard et al., 2000). Proteomics approaches to environmental stress have since become quite popular to explore changes in the relative abundance or post-translational modification status of proteins (Dowling and Sheehan, 2006; Monsinjon and Knigge, 2007). Proteomics may discover previously unknown cellular effects of environmental stressors on marine organisms or reveal effects on protein components of key metabolic pathways in response even to modest environmental stress (Tomanek, 2011, 2012). Stress response protein patterns

(protein expression signatures) induced or suppressed by toxicity can be conveniently identified with this approach (Shephard et al., 2000; Nesathyy et al 2007). This has now been applied to bivalves to support toxicological studies from a broad range of hazardous chemicals and natural toxins in this important group of animals (Dowling and Sheehan, 2006; Monsinjon and Knigge, 2007; Campos et al 2012).

Current methods for 2DE and MS analysis allow detection of alterations in levels of structural proteins, and key proteins of the oxidative stress defence mechanisms. In the present thesis, a proteomics-based 2-DE separation technique coupled with MS was extensively used for the comparative analysis of mussel proteins (see workflow in figure 10)

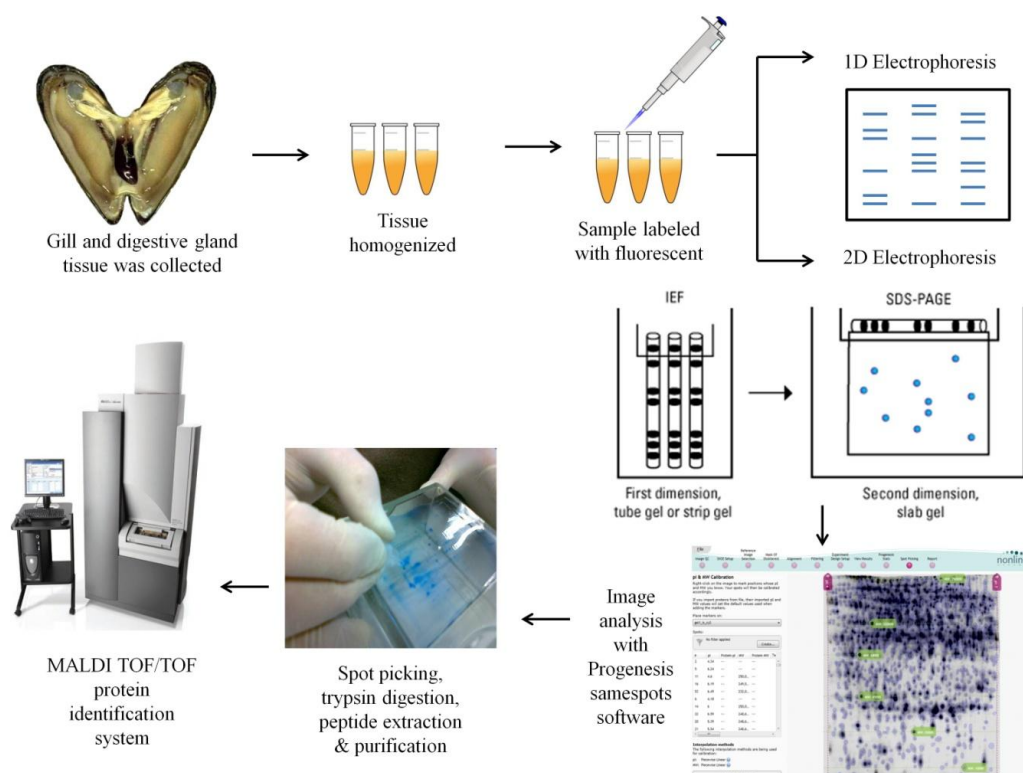


Figure 10. Work flow of proteomic work in present study.

1.6.1 Redox proteomics

Under conditions of oxidative stress, proteins absorb approximately 70% of ROS (Davies, 2005). This reflects the greater abundance of proteins compared to other oxidation targets such as DNA and lipids. This leads to a wide variety of possible structural changes to target proteins including carbonylation, thiol oxidation, methionine oxidation and hydrolysis (Davies et al., 2005; Sheehan et al., 2012). Some of these modifications are reversible by repair systems but most are not and lead to inactivation of the protein and its eventual clearance from the cell (Winterbourne, 2008; Go and Jones, 2013). Therefore oxidation substantially complicates the proteome, producing multiple versions of individual proteins that differ from each other in covalent structure, activity and cellular half-life. Importantly, these changes would not necessarily be detected from relative abundance measurements alone such as we see in protein expression signatures. It should be noted that, even in unstressed cells, redox signalling is an important part of normal cell signalling, for example altering flows of metabolites such as glucose in response to the cell's needs (Winterbourn and Hampton, 2008). Oxidation in response to external pro-oxidants can therefore also be expected to impact on redox signalling leading to toxicity by this mechanism (Go and Jones, 2013).

1.6.2 Protein thiols

Cysteine is the second least abundant residue in proteins (Winterbourn 2008). It is also anomalous on the hydrophobicity scale and is often found in the interior of proteins where it is often very well-conserved suggesting it is unusually functionally important (Marino and Gladishev, 2010). Cysteine's thiol (-SH) group performs key

roles in intracellular antioxidant defences. Cysteine residues in the active sites of proteins such as thioredoxins (Trx), glutaredoxins (Grx) and peroxiredoxins (Prx) detoxify reactive oxygen species (ROS), reactive nitrogen species and reduce oxidized protein thiols (Raquel et al 2010; Winterbourn, 2008). These, in turn, are re-reduced by reducing equivalents obtained by oxidised glutathione (GSSG) from $\text{NADPH} + \text{H}^+$ in a reaction catalysed by GSH reductase and yielding 2 GSH molecules. Oxidation and reduction of thiol proteins act as a mechanism by which reactive oxidants integrate into cellular signal transduction pathways. Thiol proteins are the best targets because cysteine residues are especially sensitive to oxidation, and changes in enzymatic activity or binding characteristics due to oxidation provide a mechanism for transmission of signals (Winterbourn and Hampton, 2013).

Figure 11 shows three pathways that may be responsible for oxidation of a protein thiol (Winterbourn and Hampton, 2013). Pathway 1: Alterations in the cellular redox buffer, GSH, results in oxidation of thiol proteins with a hierarchy dependent on the redox potential of target cysteines. Pathway 2: Direct targeting. The local environment of specific target cysteines considerably enhances their reactivity to the oxidant. Pathway 3: Facilitated targeting, a variation of direct targeting. An extremely reactive sensor protein scavenges the signalling oxidant and then facilitates oxidation of target proteins through specific protein-protein interactions and thiol transfer reactions.

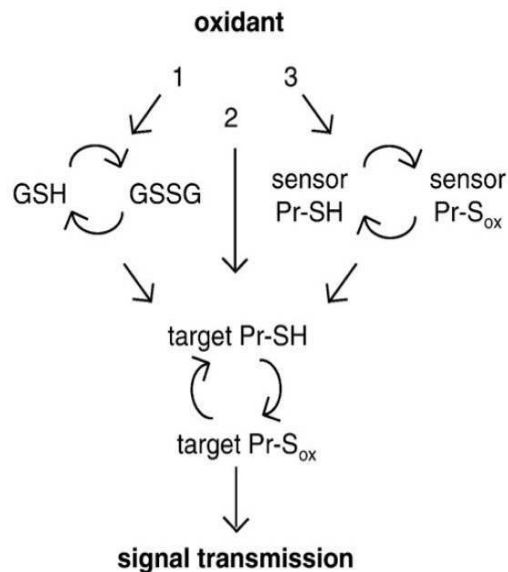


Figure 11. Redox signalling (Winterbourn and Hampton, 2013).

Cysteine is the most chemically-reactive natural amino acid and possesses functional significance in cells. GSH can form mixed disulphides (P-SS-G) with protein thiols, a process termed *glutathiolation* which is capable of targeting key proteins such as actin (Klatt and Lamas, 2000; Lind et al., 2002; McDonagh et al., 2005). Two protein -SH groups can also be oxidized to form a disulphide bond, either between separate proteins (P-SS-P) or within a single protein (P-SS) (Fig 12) (Hansen et al., 2009). Protein thiols are as quantitatively important a redox buffer resource to the cell as GSH (Hansen et al., 2009) and can sometimes be reduced after the oxidative insult to regenerate fully functional proteins (McDonagh and Sheehan 2007, 2008).

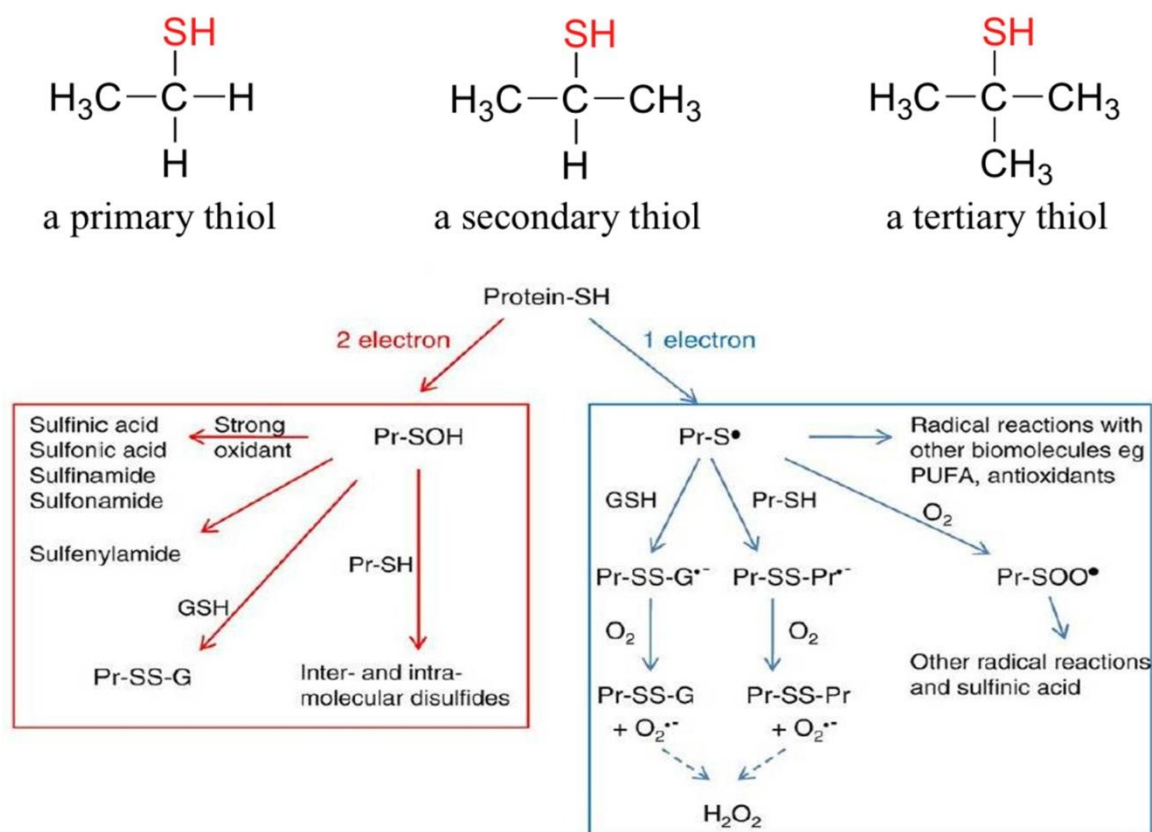


Figure 12. Modified image from Winterbourn and Hampton 2013

Oxidation of cysteine's $-\text{SH}$ group forms variously sulphenic ($-\text{SOH}$), sulphinic ($-\text{SO}_2\text{H}$), and sulphonic/cysteic ($-\text{SO}_3\text{H}$) acids, interchain/intrachain disulfide bridges ($-\text{S}-\text{S}-$), or thiolsulphinates. In intracellular stress, protein cysteines are predominantly present as free thiols ($-\text{SH}$), due to the high concentration of GSH and the high ratio of GSH/ GSSG (Hu et al., 2010; McDonagh and Sheehan, 2007). The process of oxidation of GSH to GSSG is illustrated in Fig. 13. Some protein thiols may be converted to sulphenic acid even in sub-stress conditions and this process is often associated with redox signaling (Hu et al., 2010). In fact, this oxidation and formation of disulphides are usually reversible *in vivo*. However, oxidation to $-\text{SO}_2\text{H}$ or $-\text{SO}_3\text{H}$, referred to as hyperoxidation, is usually irreversible, leading to protein degradation and clearance (Burgoyne et al 2007).

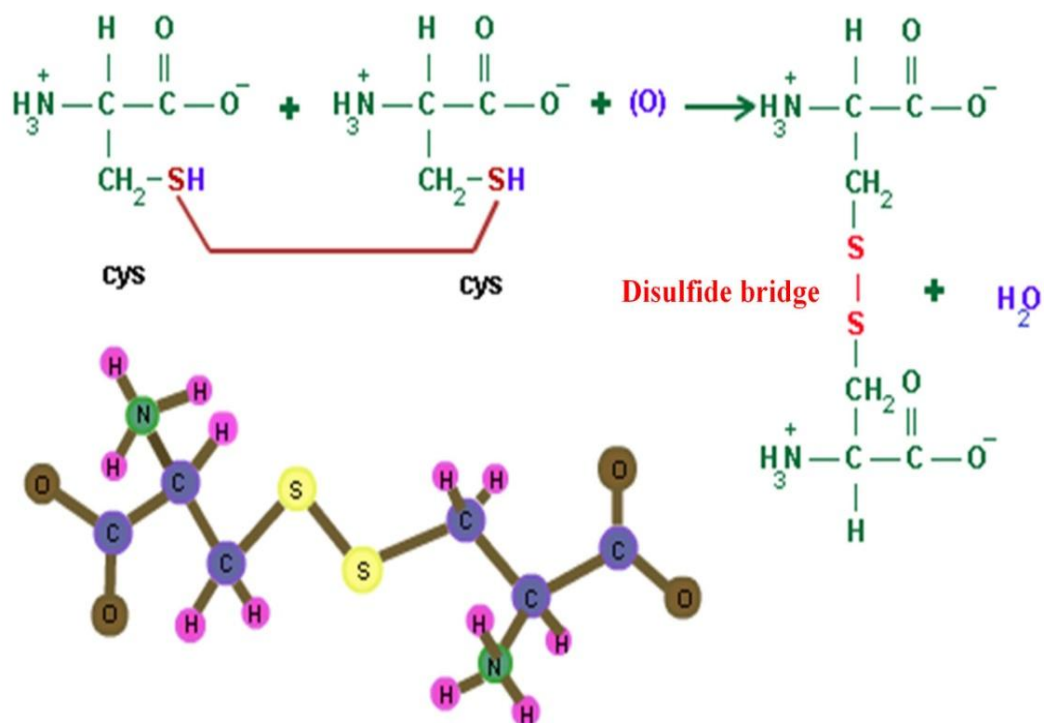


Figure 13. Disulfide bridges and oxidation-reduction. Oxidizing agent (O) provides an oxygen which reacts with the hydrogen (purple) on the -SH group to form water. The sulphurs (S) join to make the disulphide bridge. The reduction of a disulphide bond is the opposite reaction which again leads to two separate cysteine molecules (Ophardt, 2003).

Investigation of protein thiols at the cellular level, especially as biomarkers for marine pollution is still relatively under-developed. However, redox proteomics provides relatively simple and robust tools enabling us to target effects on protein thiols during oxidative stress and/or redox signalling (McDonagh et al., 2007; Hu et al., 2010; Sheehan et al., 2012)

1.7 Proposed study

1.7 Proposed study

This project aims to investigate the potential of proteomics and other biochemical approaches to detect deleterious effects of three categories of anthropogenic pollutants; respectively, a representative pharmaceutical, heavy metal and endocrine disruptor, on the popular sentinel species, *Mytilus edulis*. In the first instance, methods will be applied in a field-study comparison of a previously-investigated pair of sites within Cork Harbour, Ireland, one of which is clean (reference) and the other moderately polluted (test). Animals (minimum 3 groups of 5) sampled from the reference site will then be exposed in holding tanks to a concentration range in each case of a model endocrine disruptor (bisphenol A), heavy metal (copper) and pharmaceutical (diclofenac). Gill and digestive gland will be dissected and used as a source of protein. Extracts will be analysed for the following; Oxidation of protein thiols, effects on activity levels of important antioxidant enzymes and effects on proteomic profiles. This work will be divided into the following workpackages (WPs):

1. WP 1: Field study in Cork Harbour
2. WP 2: Effects of diclofenac, a model pharmaceutical pollutant
3. WP 3: Effects of copper, one of heavy metals substances
4. WP 4: Effects of the endocrine disruptor, bisphenol A
5. WP 5: Critical synthesis of observations and dissemination of results

1.8 Reference

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CHAPTER 2: Field study

(Protein thiols as novel biomarkers in ecotoxicology: A case study of oxidative stress in *Mytilus edulis* sampled near a former industrial site in Cork Harbour, Ireland)

Published: Journal of Integrated Omics, A methodological journal DOI: 10.5584/jiomics.v2i2.77

Protein thiols as novel biomarkers in ecotoxicology: A case study of oxidative stress in *Mytilus edulis* sampled near a former industrial site in Cork Harbour, Ireland

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Abstract

Oxidative stress produces reactive oxygen species which can modify proteins and thiols of cysteines are especially susceptible. *Mytilus edulis* was sampled from three stations in Cork Harbour, Ireland and from an out-harbour control site in Bantry Bay, Ireland. A variety of traditional biomarkers were benchmarked against thiol oxidation. Lysosomal membrane stability diminished in haemocytes from the three Cork harbour sites, although a stronger effect was observed in two in-harbour stations of environmental concern (Douglas and Haulbowline Island). Catalase and glutathione transferase (GST) activities were decreased in digestive gland extracts of animals from in-harbour sites especially the in-harbour control (Ringaskiddy) showed lower GST than Bantry. Mussels collected at Haulbowline Island showed elevated lipid peroxidation ($p < 0.05$) compared to the other three stations and decreased levels of protein thiols which is consistent with oxidative stress at this site.

Protein profiles for thiol-containing protein sub-proteomes trapped on activated thiol sepharose for each site were obtained by two dimensional electrophoresis and revealed differences between stations. Selected thiol-containing proteins were also identified by in-gel tryptic digestion and mass spectrometry; endoglucase, aginine kinase, creatine kinase 1 and endo-1, 4-beta-glucanase. Our findings confirmed that protein thiols are therefore sensitive novel biomarkers to oxidative stress.

Introduction

Cork Harbour, the second-busiest commercial port in the Republic of Ireland, is one of the world's largest natural harbours with a semi-enclosed area of approximately 25 km² [1] (Fig. 1). The harbour accepts anthropogenic inputs from industry, shipping, agricultural run-off and human sewage from the surrounding catchment of some 400,000 inhabitants and the underlying geology makes the water-body especially susceptible to pollution [2]. The intertidal area is an internationally-important wetland site for wintering waterfowl and is designated as a special protection area under the EU Birds Directive [3]. Although not extensively polluted by international standards [4], there is some localised build-up of PAHs, especially at Douglas estuary [5, 6, 7]. There is intense local and national concern that industrial activities pose an ongoing threat to the quality of the aquatic environment of the harbour and a former steel plant on Haulbowline Island is the site of a major industrial remediation project. We have previously used protein biomarkers such as glutathione transferases (GSTs) [8, 9] and heat shock proteins [9] to assess the environmental stress-status of *Mytilus edulis* sampled from the harbour. Comet assays revealed PAH-mediated genotoxicity from sediment sampled around the harbour in turbot and clam [10]. More recently, we have used proteomic methods [11, 12] to extend these studies [13-16]. In the present investigation we have explored a novel redox proteomic method based on oxidation of protein thiols due to oxidative stress benchmarked against traditional biomarkers to assess the quality of mussels at sites adjacent to Haulbowline Island. We compared these to a reference site within the harbour (Ringaskiddy) and in Bantry Bay, a deep near-pristine inlet further along the West Cork coast. Our findings suggest that mussels from the two in-harbour test sites are under some environmental stress.

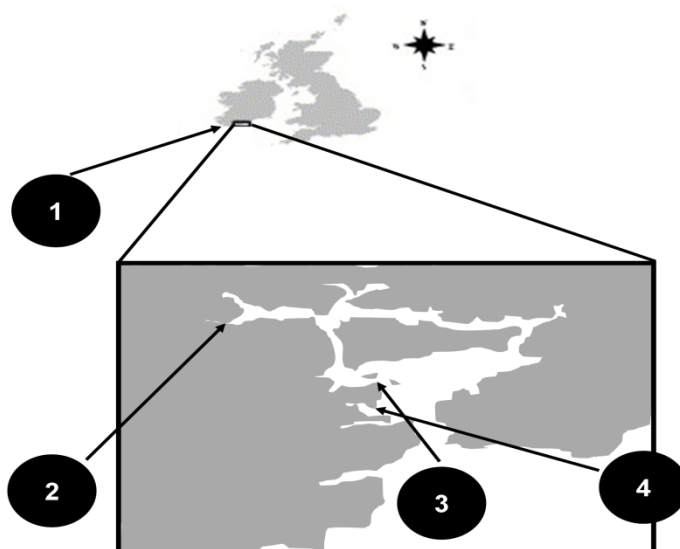
Materials and methods

Site selection and animals

M. edulis were collected from four sites in Ireland: Bantry Bay (9° 30'W, 51°40'N) and three sites within Cork Harbour; (Fig.1): Douglas (8° 23'W, 51°52'N), Haulbowline (8°17'W, 51°50'N), Ringaskiddy (8°18'W, 51°49'N). Bantry Bay is a clean area considered an appropriate control [17] which is an important site for commercial mussel aquaculture. The three Cork Harbour sites were chosen, respectively, because of presence of PAHs [Douglas; 5-7], the presence of large amounts of iron and other pollutants at a former steel plant presently undergoing remediation (Haulbowline) and previous history as an appropriate in harbour control

Figure 1. Sampling sites for *Mytilus edulis* around Cork harbour, Ireland: (1) Bantry Bay (out-harbour control site), (2) Douglas, (3) Haulbowline, (4) Ringaskiddy (in-harbour control site).

(Ringaskiddy; [9]). Thirty mussels (5-6 cm in length) were collected on a single day from the intertidal zone at low tide at the four sampling sites. Haemolymph was withdrawn using a 21 Gauge syringe from the adductor muscle of 10 animals for each site on the day of collection to measure lysosomal membrane stability in the haemocytes [18]. Digestive gland tissues were dissected, pooled (5 organisms per replicate), frozen in liquid nitrogen and stored at -80 °C.



Chemicals

Acetonitrile, bovine serum albumin (BSA), 1-chloro-2,4-dinitrochlorobenzene (CDNB), dimethyl sulfoxide (DMSO), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 5'-iodoacetamide uorescein (IAF), 1-methyl-2-phenylindole, neutral red, phenyl-methylsulphonyl uoride (PMSF), reduced glutathione (GSH), 1,1,3,3- tetramethoxypropane, and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Activated thiol sepharose (ATS) was purchased from GE Healthcare (Little Chalfont, Bucks, UK).

Sample preparation

Digestive glands were homogenized in a motor-driven Teflon Potter-Elvehjem homogenizer in 10 mM Tris H-Cl, pH 7.2, containing 500 mM sucrose, 1 mM EDTA and 1 mM PMSF. Supernatants were collected by centrifugation at 20,000 ×g

and stored at -70 °C until required for analysis. Protein content was calculated by the method of Bradford using BSA as a standard [19].

Lysosomal membrane stability

Lysosomal membrane stability was measured by the neutral red retention time assay [NRRT; 18]. Haemocytes from the adductor muscle were incubated on a glass slide with a freshly prepared neutral red (NR) working solution (2 µl/ml saline from a stock solution of 20 mg neutral red dye dissolved in 1 ml of DMSO) and microscopically examined at 15 min intervals to determine the time at which 50% of cells had lost to the cytosol the dye previously taken up by lysosomes.

Antioxidant enzymes

Catalase activity (CAT) was measured according to the method of Aebi [20]. This method is based on measuring decrease in absorbance at 240 nm due to the consumption of hydrogen peroxide (H₂O₂). Activity was expressed as U/min/ mg protein $\epsilon = -0.04 \text{ mM}^{-1} \text{ cm}^{-1}$). GST activity was determined using CDNB as substrate [21]. The reaction rate was detected at 340 nm, and expressed as nmol CDNB conjugate formed/ min/mg protein $\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Lipid peroxidation

Lipid peroxidation was measured by determining malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids. It was determined in samples homogenized (1:3 w/v) in 20 mM Tris-HCl pH 7.4, centrifuged at $3,000 \times g$ for 20 min and then derivatized in a 1 ml reaction mixture containing 10.3 mM 1-methyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 µl water and an equal volume of sample or standard (standard range 0–6 µM 1,1,3,3-

tetramethoxypropane, in 20 mM Tris-HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at $15,000 \times g$ for 10 min and read spectrophotometrically at 586 nm; levels of MDA were calibrated against an MDA standard curve and expressed as nmol/g wet weight [22].

Labelling protein thiols

Protein thiols present in protein extracts were labelled by adding IAF in DMSO to a final concentration of 800 μ M and incubating at room temperature for 2 h in the dark. IAF reacts specifically with reduced thiols (-SH) but not with oxidised variants such as sulphenic acid (-SOH) or disulphides (-S-S-) which might be expected to form on oxidative stress [23].

Protein electrophoresis

Proteins were resolved using one-dimensional electrophoresis (1DE) in 12% polyacrylamide gels [24]. Samples were diluted in buffer lacking β -mercaptoethanol, to avoid reduction of disulphide bridges. Gels were scanned in a Typhoon 9400 scanner (GE Healthcare, UK; excitation, 490-495 nm; emission, 515-520 nm) and were subsequently stained with Coomassie G250. Equal amounts of protein were loaded in 12 wells (3 replicates for each treatment) and repeated at least 3 times. Two-dimensional SDS PAGE electrophoresis (2DE) analysis was performed on protein extracts trapped by covalent disulphide exchange on ATS [25-27]. Protein samples were precipitated with TCA/acetone and re-suspended in rehydration buffer containing 5M urea, 2 M thiourea, 2% CHAPS, 4% ampholyte (Pharmalyte 3-10, Amersham-Pharmacia Biotech, Little Chalfont, Bucks., UK), 1% Destreak reagent (Amersham-Pharmacia Biotech) and trace amounts of bromophenol blue. A final volume of 125 μ l was loaded on 7cm IPG strips pH 3-10NL on the bench overnight.

Proteins were focused on a Protean IEF Cell (Biorad) with linear voltage increases: 250 V for 15 min; 4000 V for 2h; then up to 20,000 Vh. After focusing, strips were equilibrated for 15 min in equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% DTT and then for 15 min in equilibration buffer containing 2.5% iodoacetamide. Equilibrated strips were electrophoresed on 12% SDS-PAGE gels at a constant voltage (150 V) at 4 °C. 2D gels were scanned by calibrated densitometer (Bio-Rad Laboratories) of gels visualized by Colloidal Coomassie staining [28].

Quantification of proteins

For each 1DE gel, bands detected by the Typhoon 9400 scanner, were subsequently analyzed by Quantity One image analysis software (Bio-Rad, Hercules, CA, USA) measuring the total intensity for each lane, quantified as arbitrary units (A.U.). 1DE gels stained with Coomassie blue G250 were scanned in a GS-800 calibrated densitometer and total optical density of each lane measured by Quantity One image analysis software. Total optical densities for each lane were normalized with those from coomassie staining for the same gel track.

In-gel digestion and MALDI-TOF/TOF analysis

Proteins were excised from gels and cleaved with trypsin by in-gel digestion. The protein spot digestion was performed in OMX-S devices according to the manufacturer's instructions (OMX, Munich, Germany). Briefly, 20 µl of modified trypsin (10 ng/µL in 50mM ammonium bicarbonate) were added to the device and centrifuged briefly at 3,800 x g. The digestion procedure was carried out at 50°C for 45 minutes with gentle agitation. The peptide solution was removed from the reactor compartment by centrifugation at 1,000 x g for 3 min. Peptide solutions were

desalted and concentrated with chromatographic micro columns using GELoader tips packed with POROS R2 (Applied Biosystems, Foster City, California, USA; 20 μ m bead size) and then directly eluted onto the MALDI target plate using 0.5 μ l of 5 mg/ml α -ciano-4-hydroxy-trans-cinnamic acid (α -CHCA) in 50% (v/v) ACN with 2.5% (v/v) formic acid and air-dried.

Tandem mass spectrometry analysis was performed using a MALDI-TOF/TOF 4800plus mass spectrometer (Applied Biosystems). The equipment was calibrated using angiotensin II (1,046.542 Da), angiotensin I (1,296.685 Da), Neurotensin (1,672.918 Da), adrenocorticotrophic hormone (ACTH) (1-17) (2,093.087 Da), and ACTH (18-39) (2,465.199) (Peptide Calibration Mixture 1, LaserBio Labs, Sophia-Antipolis, France). Each reflector MS spectrum was collected in a result-independent acquisition mode, typically using 750 laser shots per spectra and a fixed laser intensity of 3,200V. The fifteen strongest precursors were selected for MS/MS, the strongest precursors being fragmented first. MS/MS analyses were performed using collision induced dissociation (CID) assisted with air, with collision energy and gas pressure of 1 kV and 1 x 10⁶ torr, respectively. Each MS/MS spectrum collected consisted of 1,200 laser shots using a fixed laser intensity of 4,300V.

Protein identification

Protein identification was performed using MASCOT (version 2.2; Matrix Science, Boston, MA) search engine. Searches were performed using combined analysis of the intact masses of the tryptic peptides (MS) and tandem mass data (MS/MS). Search parameters were set as follows: minimum mass accuracy of 50 ppm for the parent ions, an error of 0.3 Da for the fragments, two missed cleavages in peptide masses, and carbamidomethylation (C), oxidation (M), deamidation (NQ), Gln-

>pyro-Glu (N-term Q) were set as variable amino acid modifications and a non-redundant NCBI database (released 2012_01) was used. Peptides were only considered if the ion score indicated extensive homology ($p < 0.05$). Proteins were considered if having significant MASCOT score and at least one peptide with extensive sequence homology. Automated GO annotation was performed using the GO categories of the best hit derived from the BLASTp results (BLASTp minimal expectation value set to 1×10^{-3}) for additional information on functional pathway.

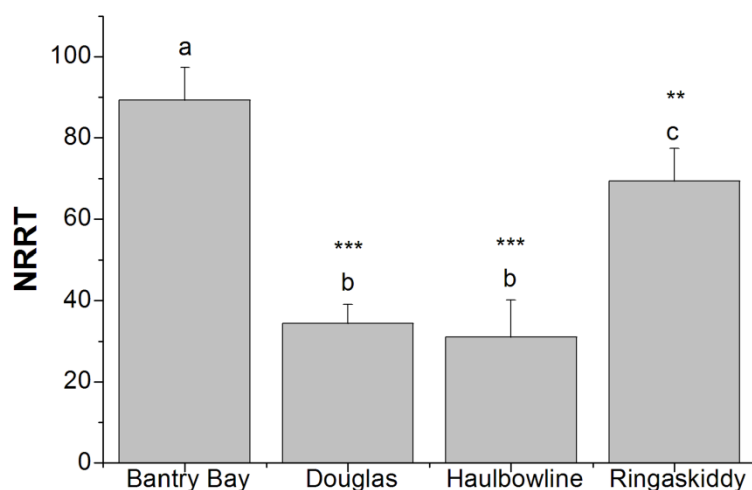
Statistical analysis

All data are means \pm standard deviation (SD) of triplicate determinations on three independent extracts for each treatment studied. Statistical analyses of data were performed using the Software Statistica 7.0 (Stat Soft, Tulsa, Oklahoma, USA). Samples were tested using one-way ANOVA, homogeneity of variance was tested by Cochran C and mathematical transformation applied if necessary; post hoc comparison (Newman–Keuls) was used to discriminate between means of values. Differences were considered statistically significant when $p < 0.05$.

Results

Lysosomal membrane stability

Lysosomal membrane stability measured in freshly sampled haemocytes showed lower NRRT at all three stations compared to the out-harbour control site, Bantry Bay. The NRRT observed in mussels from Douglas and Haulbowline were less than half that for Bantry while Ringaskiddy was approximately 80% of Bantry (Fig.2).



Antioxidant enzymes

CAT activity was significantly lower ($p < 0.05$) in mussels collected from the three in-

Figure 2. Lysosomal membrane stability measured as neutral red retention time (NRRT; min) in haemolymph from *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean \pm SD. Superscripts of different letters are significantly different from each other at ** $p < 0.01$; *** $p < 0.005$. $n = 5$

harbour stations compared to Bantry Bay (Fig.3). Significantly lower GST activity was found in mussel digestive glands at the three in-harbour stations compared to Bantry Bay (Fig.4).

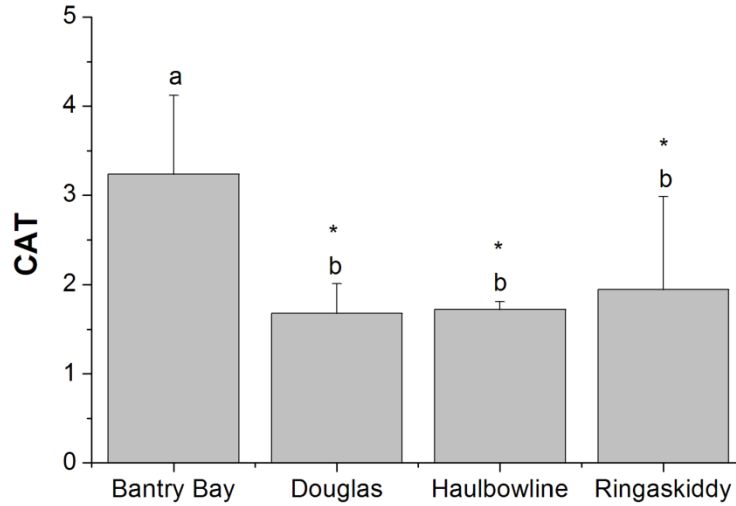


Figure 3. Catalase activities (CAT; U/min/mg prot) in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean \pm SD. Super- scripts of different letters are significantly different from each other at * $p < 0.05$. $n = 5$

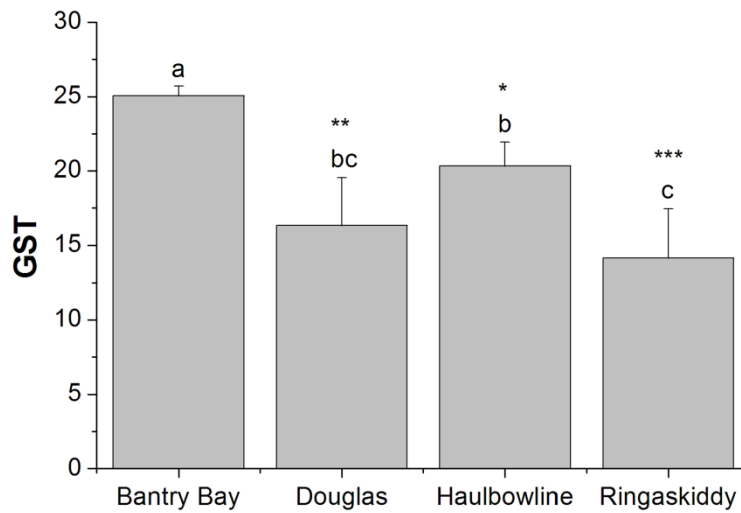


Figure 4. Glutathione transferase activities (GST; nmol CDNB conjugate formed/min/mg prot) in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean \pm SD. Superscripts of different letters are significantly different from each other at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$. $n = 5$

Lipid peroxidation

The highest MDA levels were determined in digestive gland of mussels collected at Haulbowline, showing higher lipid peroxidation ($p < 0.05$) compared to the out-harbour control site, Bantry Bay (Fig.5).

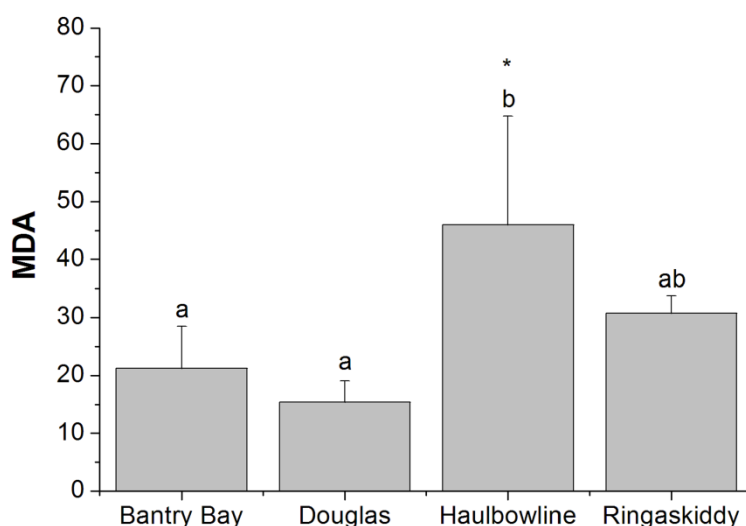


Figure 5. Lipid peroxidation measured as malondialdehyde (MDA) levels (nmol/g wet weight tissue) in digestive gland of *M.edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean \pm SD. Superscripts of different letters are significantly different from each other at $*p < 0.05$. $n=5$

Protein thiols

1DE separation of IAF-labelled proteins revealed a decrease in total thiol-containing proteins in samples from Haulbowline ($p=0.03$) and Douglas ($p=0.052$) compared to Bantry Bay (Fig.6). Thiol-containing proteins were trapped on ATS [25-27] and separated by 2DE (Fig.7). This revealed differences in spot patterns which we attribute to oxidation of thiols in specific proteins supporting the results obtained by

1DE. Taken together, these data suggest that protein thiols decreased strongly in digestive gland of mussels collected in Haulbowline followed by Douglas.

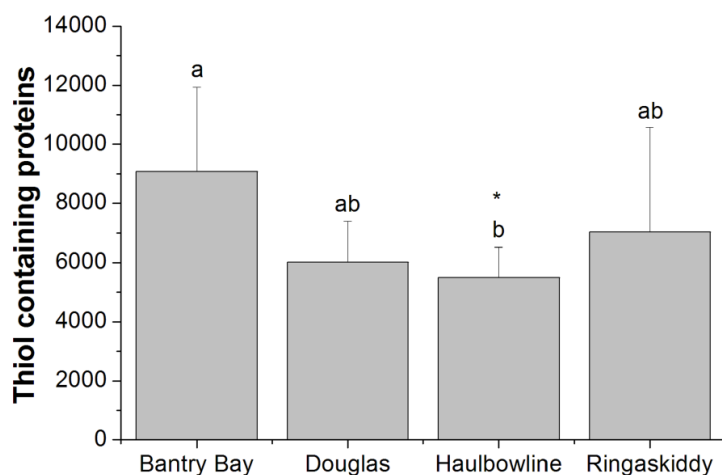


Figure 6. 1DE of thiol-containing proteins (normalized by total protein amounts) in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed in A.U. as mean \pm SD. Superscripts of different letters are significantly different from each other at $*p < 0.05$. $n = 5$

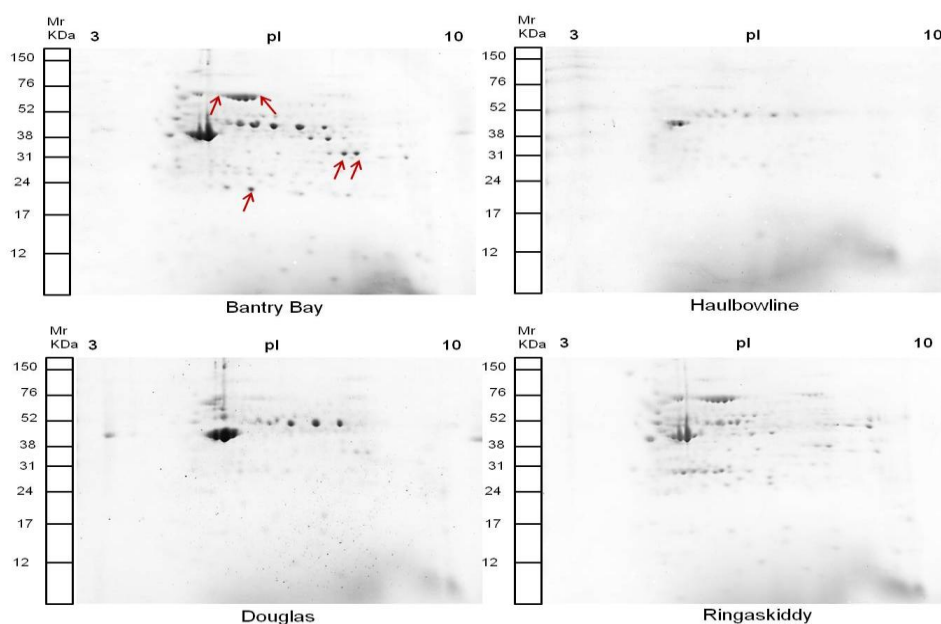


Figure 7. Representative 2DE of thiol containing proteins trapped by ATS in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Spots present in Bantry Bay and/or Ringaskiddy 2DE but weakened or missing in Douglas and Haulbowline are shown with arrows. These spots were selected for the protein identification.

Identified proteins

The weakened or missing spots from 2DE of samples collected in Haulbowline and Douglas were selected for protein identification (Fig.7). The reason is that these thiol-containing proteins were unable to be selected by ATS due to oxidation of cysteines. Because of the lack of a full genome sequence for *Mytilus* species it was necessary to search other species for matches with peptides derived from in-gel digestion of selected spots. Four of the selected proteins were successfully identified in this way showing significant MASCOT scores and at least one peptide with extensive sequence homology (Table 1).

The MS results confirmed that all selected spots were thiol-containing proteins but only one of them, endo-1,4-beta-glucanase (spot 4), was found to correspond to an *M. edulis* sequence. Spot 1 was identified as another endoglucanase but from *Mizuhopecten yessoensis*, a scallop belonging to the family of Pectinidae but to the same class of Bivalvia as *M. edulis*. Spot 2 matched well with arginine kinase from *Conus novaehollandiae*, a marine gastropod mollusc belonging to the same Phylum (Mollusca) as *M. edulis*. Spot 3 was similar to creatine Kinase 1 of *Lethenteron camtschaticum*, a freshwater fish not taxonomically similar to the blue

mussel but included because of its high MASCOT score and good expectation value (Table 1).

Table 1. Identified proteins in digestive gland of *M. edulis* by MALDI TOF MS. Cut-off MASCOT score ($p < 0.05$) = 84.

Spot #	Accession #	Protein ID	Organism	Theoretical/observed pI	Theoretical/observed Mr (kDa)	Mascot score	Expectation value	Peptides ID	Function
1	gi 254553092	Endoglucanase	<i>Mizuhopecten yessoensis</i>	5,66/~5.5	64.152/52-76	120	1.5e-0.005	1	Carbohydrate metabolism
2	gi 301341836	Arginine kinase	<i>Conus novae-hollandiae</i>	6,34/~6.4	39.379/31-38	135	4.8e-007	1	Energy metabolism
3	gi 42627683	Creatine kinase 1	<i>Lethenteron camtschaticum</i>	6,71/~6.8	42.620/31-38	166	3.8e-010	1	Energy metabolism
4	gi 12230122	Endo-1,4-beta-glucanase	<i>Mytilus edulis</i>	6,79/~6	19.699/17-24	582	9.6e-052	5	Carbohydrate metabolism

Discussion

Cysteine is the second least-abundant residue in proteins and is the main point of crosstalk between redox status and cell signalling [29]. Both in controlled exposure experiments with pro-oxidants in holding tanks [30] and in the field [31], we have previously shown that mussels change aspects of their thiol chemistry in response to oxidative stress. Because of their roles in buffering transient increases in ROS [32] and in cellular redox signaling pathways [29], protein thiols are especially attractive targets as possible novel biomarkers for oxidative stress. A multiplexing approach allowed simultaneous determination of total thiols and total protein in electrophoretic separations by exploiting the specificity of IAF for reduced thiols was performed in this study and decreased IAF labeling was attributed to thiol oxidation [23]. The

Haulbowline site showed decreased total thiols suggesting that proteins, as well as lipids, experienced attack by ROS at this site. This observation was extended by trapping thiol-containing proteins on ATS and analyzing the thiol-proteome by 2DE [26-28]. This revealed closely-comparable separations in samples from each site but with individual spot differences, consistent with differences in thiol status across the sub-proteomes. *M. edulis* is a sentinel species widely-used in surveillance of environmental quality with particular relevance to marine estuaries [33, 34]. A number of biomarkers useful for assessing environmental quality have been developed in mussels [35, 36] but we are interested in identifying novel protein biomarkers that may complement these traditional indices and possibly yield greater insights to toxicity mechanisms [9, 13-17]. In the present case study, two sites of environmental interest within Cork Harbour, Ireland, were compared to in-harbour and out-harbour reference sites by measuring physiological and bio-chemical indices of stress, mainly in digestive gland, a key site of enzymatic detoxification [37]. Haemocyte NRRT was decreased in the two in-harbour test sites while lipid peroxidation was significantly increased in the Haulbowline site. This is consistent with significant environmental stress at that location possibly arising from residual pollutants from recently-removed slag heaps in a former steel plant currently undergoing remediation.

Lower levels of CAT and GST activities at both sites are consistent with these findings since these enzymes contribute strongly to defence against xenobiotic and oxidative stress [38-40]. CAT detoxifies H_2O_2 to water in vivo but, in the presence of iron, H_2O_2 produces the hydroxyl radical by means of the Fenton reaction [29]. H_2O_2 is formed naturally in water as a result of photo-oxidation [41]. Enhanced CAT activity has been reported in fish and invertebrate species [42, 43]

and its inhibition has been suggested as a transitory response to acute pollution [36]. Decreased CAT activity, combined with available iron, could potentially result in formation of the hydroxyl radical leading to oxidative stress.

GST is a phase II detoxification enzyme that catalyses conjugation of electrophiles to GSH [40] which has also found use as a biomarker in mussels [9, 17]. In this study, all three Cork Harbour sites showed significantly lower GST levels than Bantry Bay. GST contributes to protecting tissues from oxidative stress by catalytic detoxification and binding as it is inducible by a wide range of chemical agents, some of which are also substrates such as hydroperoxides [40]. Increase of GST activity can therefore be due to increased detoxification of hydroperoxides. GSH conjugates are subsequently enzymatically degraded to mercapturates and excreted. However, under more intense or prolonged oxidative stress conditions, GST catalytic activity can be compromised due to conjugation of GSH to xenobiotic electrophilic centres causing a depletion of GSH and GST inhibition [44]. Generally, GST activity is lower in mussel digestive gland than gill [17]. We have previously found that gill GST activity varies with pollution status [9, 17]. However, while GST activity of *M. edulis* digestive gland did vary between sites on the South Coast of Ireland [17] it did not vary in digestive gland of *M. galloprovincialis* sampled from polluted sites in Venice Lagoon [8]. This suggests that digestive gland GST level may not be as responsive to pollution as gill.

MDA level is one of the oxidative stress parameters that has been measured in bivalve molluscs, especially in mussels, to investigate the biomarker's response to cellular free radical toxicity under metal exposure [45]. Digestive glands of mussels collected at Haulbowline showed statistically higher MDA levels than those sampled from Bantry Bay ($p = 0.046$) but not versus Ringaskiddy (in-harbour control).

Intriguingly, no relevant effects of lipid peroxidation or decrease of amount of thiol-containing protein were observed in mussels collected in Douglas. Our results showed that MDA levels in digestive gland of mussels collected at Haulbowline were statistically higher than those sampled from Bantry Bay ($p= 0.046$) but not versus Ringaskiddy (in-harbour control). Intriguingly, no relevant effects of lipid peroxidation or decrease of amount of thiol-containing proteins was observed in mussels collected in Douglas.

These data suggest that protein thiols play a role in protection against lipid peroxidation. Oxidation of protein thiols, usually occurs by two different mechanisms: (1) lipid peroxidation induced by the depletion of GSH generates reactive aldehydes [46, 47] which may react with protein thiols; (2) reactive metabolites may react directly with protein thiols. Metals and their chelate complexes, such as copper, chromium, nickel, and cadmium, are implicated in lipid peroxidation [48]. It is likely that organic compounds (e.g. PAH, PCB) or metals released to seawater from the former steel plant on Haulbowline Island affected the local intertidal *M. edulis* population. To our knowledge, this report is the first to show redox cysteine modifications in endoglucanase, arginine kinase and creatine kinase proteins in *M. edulis*.

Endoglucanases are enzymes belonging to the cellulose family involved in carbohydrate metabolism. The endoglucanases identified are rich in cysteine residues and endo-1, 4-glucanase which has been sequenced and cloned in digestive gland of *M. edulis* [49,50] contains twelve cysteine residues involved in six disulfide bonds. It is thought that these disulphides may contribute to anti-freezing properties of this protein at low temperature. Decrease of cellulase activity has been considered as one of the major potential biomarkers for exposure to pesticides in aquatic invertebrates

[51]. However De Coen et al. (2001) have found that relationships between enzymatic endpoints in carbohydrate metabolism and population level effects observed in *Daphnia magna* were toxicant-specific and no single enzyme in carbohydrate metabolism could predict quantitative changes in population characteristics [52].

Arginine kinase catalyzes the reversible transfer from phospho-L-arginine to ADP to form ATP and is important for buffering ATP levels during burst muscle contraction in invertebrates [53]. Our results showed that this enzyme was down-regulated in Douglas and especially Haulbowline. This enzyme has been proposed to increase the ability of invertebrates to cope with the stress of variable environmental conditions related to hypoxia and acidosis [54, 55]. Silvestre et al. (2006) found strong down-regulation of this enzyme in the gills of the Chinese mitten crab after chronic cadmium exposure [56]. We also found that creatine kinase is another thiol-containing protein that may be sensitive to oxidative stress. It is a key enzyme in energy metabolism catalyzing reversible phosphorylation of creatine by ATP [55]. Sethuraman et al. (2004) showed that cysteine thiols of sarcomeric creatine kinase were oxidized after exposure to high concentrations of hydrogen peroxide [57]. A previous study showed that arginine and creatine kinase are sensitive to oxidation although by different mechanisms. Mammalian creatine kinase was very sensitive to the superoxide radical resulting in loss of enzyme activity whereas arginine kinase was less affected by comparable exposure [58]. These authors also found that loss of creatine kinase activity can be due to its high susceptibility to hypoxic conditions. The increase of nutrients usually found in polluted areas like some harbours [12, 59, 60] can be the result of algal bloom and subsequent hypoxia and ROS production enhance.

Taken together, our results show that mussels from the Haulbowline site experience considerable physiological and oxidative stress, which is consistent with the presence of pollutants originating from the nearby former steel plant. We suggest that protein thiols may be a potentially useful novel biomarker for oxidative stress in environmental toxicology.

Acknowledgements

Our laboratory is supported by the Programme for Research in ird Level Institutions of the Higher Education Authority of Ireland. ST received an EMBARK award from the Irish Research Council for Science Engineering and Technology.

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CHAPTER 3:

(Redox proteomic analysis of *Mytilus edulis* gills: Effects of the pharmaceutical diclofenac on a non-target organism)

Published: Drug Testing and Analysis Journal. DOI: 10.1002/dta.1786

**Redox proteomic analysis of *Mytilus edulis* gills: Effects of the pharmaceutical
diclofenac on a non-target organism**

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Abstract

Veterinary and human pharmaceuticals are an emerging category of chemical pollutants with potential to cause serious toxicity to non-target organisms. Filter-feeding aquatic organisms such as mussels are especially threatened. In this study, the blue mussel, *Mytilus edulis*, was exposed to two doses (0.2mg/l and 1mg/l) of the anti-inflammatory diclofenac. Effects on the gill, the principal feeding organ of mussels, were investigated. It was noted that, while no effect was evident on gill glutathione transferase or catalase activities, there was a tissue-specific increase in glutathione reductase activity and reduction in total protein thiol groups. Two dimensional electrophoresis was performed and some affected proteins identified by in-gel tryptic digestion and peptide mass fingerprinting. Of these, four unique proteins (caspase 3/7-4, heat-shock cognate protein 70, a predicted enolase-like protein, arginine kinase) were found to be oxidised whilst eight unique proteins (β -tubulin, actin, isocitrate dehydrogenase, arginine kinase, heavy metal-binding HIP, cytosolic malate dehydrogenase, proteasome subunit alpha type 2, Mg:bb02e05 and superoxide dismutase) were found to have altered abundance. In addition, bioinformatic analysis suggested putative identities for six hypothetical proteins which either were oxidised or decreased in abundance. These were; 78kDa glucose-regulated protein precursor, α -enolase, calreticulin, mitochondrial H⁺-ATPase, palmitoyl protein thioesterase 1 and initiation factor 5a. It is concluded that diclofenac causes significant oxidative stress to gills and that this affects key structural, metabolic and stress-response proteins.

Introduction

Pharmaceuticals developed for veterinary and human applications represent an important emerging category of chemical pollutants with potential to cause toxicity in non-target organisms [1]. Their amounts in the environment increase year-on-year due to an ageing and growing human population and more intensive agricultural practices all of which contribute to increased use of many categories of pharmaceuticals. By definition, pharmaceuticals are often stable in environmental compartments and are usually developed to exert effects on specific biochemical targets at low doses. This means that they may retain potential for bioactivity, even after deposition in the environment. In addition, the conservation of biochemical targets between humans and animals on the one hand, and non-target organisms on the other is likely to lead to non-target effects [2]. There is now significant concern internationally about the potential environmental implications of pharmaceuticals developed for humans and agricultural animals on non-target species [1]. Filter-feeding aquatic organisms such as mussels are of special interest in this regard, since they filter estuarine water very efficiently and are known to be capable of bioconcentrating pharmaceuticals and other xenobiotics in their tissues [3]. Exposure of *Mytilus edulis trossulus* in the Baltic Sea to a panel of three pharmaceuticals revealed an ability in this organism to bioconcentrate these compounds in tissues by two to three times the levels found in effluents from sewage plants [4]. Bivalves represent an important destination for pharmaceuticals emitted from sewage plants [5] with possible implications for human health where shellfish are an important dietary component for human populations. From an analytical chemistry perspective, detection of pharmaceuticals and their metabolites in mussel tissues may be especially useful in environmental surveillance of pharmaceuticals [5].

Amongst pharmaceuticals there is particular interest in the non-steroidal anti-inflammatory diclofenac, which was recently included in the European Union's list of priority substances [6]. Many pharmaceuticals and their derivatives can trigger oxidative stress in bivalves as part of their toxicity which is amenable to investigation by redox proteomics methods [7, 8]. We previously explored the effects of diclofenac on digestive gland of the blue mussel, *M. edulis* [7]. However, the gill is the principal feeding organ of this animal and this organ also is an important site for drug detoxification [9]. Evidence for changes in antioxidant enzyme profiles has previously been obtained for the gills of *M. galloprovincialis* [6]. In the present study, we have explored further the effect of diclofenac exposure on detoxification and antioxidant enzyme activities, protein thiol redox status and protein expression profiles obtained from two-dimensional electrophoresis (2-DE) in gills of *M. edulis*. Mass spectrometry (MS) identification by peptide mass fingerprinting made possible identification both of oxidised proteins and proteins whose abundance was altered. These identifications offer insights into the biochemical consequences of diclofenac toxicity in this important non-target species.

Materials and methods

Chemicals

Bovine serum albumin (BSA), dimethyl sulphoxide (DMSO), diclofenac, Tris-HCl, 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), EDTA, phenyl-methylsulphonylfluoride (PMSF), reduced glutathione (GSH), oxidised glutathione (GSSG), 5-(iodoacetamido) fluorescein (IAF) and diclofenac were purchased from Sigma Aldrich.

Animals and experimental design

Mytilus edulis were collected in March 2013 from a clean site in Bantry Bay, Ireland (9° 30'W, 51°40'N). This area is well-known for commercial mussel aquaculture [10]. One hundred mussels (5-6 cm in length) were collected from the intertidal zone at low tide and transported to the laboratory. The mussels were cleaned and acclimated for 7 days in artificial sea water (ASW) at 10° C (\pm 1° C) with a 12-h light/dark cycle. Mussels were fed daily with a commercial solution (Shellfish diet 1800) containing a mixture of microalgae; *Isochrysis sp*, *Pavlova sp*, *Thalassiosira weissflogii*, and *Tetraselmis sp* at the recommended 2% maximum feed conversion rate during acclimation and exposure. The animals were exposed to two concentrations of diclofenac (0.2 mg/l and 1.0 mg/l, respectively) with daily water change. Diclofenac was dissolved in DMSO added to the tanks after water change, to a final concentration of 0.001%. Four groups were established for the experiment: control mussels (ASW only); solvent control (ASW+ 0.001% DMSO); 0.2 mg/l (ASW+DMSO+ diclofenac); 1mg/l (ASW+DMSO+ diclofenac). The tank was filled

with 2L of artificial sea water (ASW) and one-hundred animals were used for this experiment. Each of the groups contained 5 biological replicates, each consisting of 5 pooled animals. The mussels were fed for 4 hours prior to exposure. Water was changed and the mussels then were exposed to the drugs for a period of 7 days. In addition, mussels were then allowed to recover for a further 7 days before tissues were dissected.

Tissue preparation

Gills were dissected from all groups and 5 animals in each replicate group were pooled together (n=100). The samples were homogenized in a motor-driven Teflon Potter-Elvehjem homogenizer in Tris-HCl buffer containing 10 mM Tris-HCl , 0.5 M sucrose, 0.15 M KCl, 1mM EDTA and 1mM PMSF with a pH 7.2 at a weight: volume ratio of 1:3. The homogenate was centrifuged at 15 000 g for 1h at 4°C and the supernatant was collected and stored in -80°C for further analysis. The protein content was estimated by the Bradford method using BSA as a standard [11].

Enzyme assays

Glutathione transferase (GST) activity was determined using CDNB as substrate [12]. The reaction rate was detected at 340nm and specific activity was expressed as nMol CDNB conjugate formed/min/mg protein; $\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Glutathione reductase (GR) activity was determined using β -NADPH as substrate [13]. The reaction rate was detected at 340nm and specific activity was expressed as nMol NADPH consumed/minute/mg protein; $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. Catalase activity (CAT) was measured according to the method of Aebi [14]. This method is based on

measuring decrease in absorbance at 240 nm due to the consumption of hydrogen peroxide (H₂O₂). Specific activity was expressed as U/min/mg protein; $\epsilon = 0.04 \text{ mM}^{-1} \text{ cm}^{-1}$.

Fluorescent protein labelling and one-dimensional electrophoresis

IAF was added directly to homogenates (150 μg protein) from a 20 mM stock to a final concentration of 0.2 mM and incubated on ice in the dark for 2 h. This compound reacts specifically only with reduced thiols (-SH) but not with oxidised variants such as sulphenic acids (-SOH) or disulphides (-S-S) which might be expected to form on oxidative stress [15]. Fluorescently-labelled proteins were resolved using one-dimensional electrophoresis in 12% polyacrylamide gels, at a loading of 30 μg per lane, with four replicate lanes per sample [16]. Briefly, proteins were dissolved in sample buffer (dH₂O; 0.5 M TRIS-HCl, pH 6.8; glycerol; 10% SDS; 0.5% bromophenol blue), and electrophoresed (4°C) at 120V for two hours in running buffer (10X stock: 30.0g Tris base; 144.0g glycine; 10.0g SDS; 1L H₂O) in 12% polyacrylamide gels. After electrophoresis, gels were scanned in a Typhoon scanner, model 9410 (Amersham Biosciences) with excitation wavelength set at 488 nm and emission wavelength at 520 ± 20 nm filter. The gels were then stained with colloidal coomassie [17]. After destaining, gels were scanned in a calibrated densitometer (BioRad, Hercules, CA, USA).

Two-dimensional electrophoresis

Homogenates (300 μg protein) were labelled with IAF as stated in the previous section. Protein pellets (150 μg protein) were resuspended in 125 μL rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 1.2% bis (2-hydroxyethyl)-disulfide, 4% ampholytes (3-10 for IEF, GE Healthcare) and a trace of bromophenol blue [18]. The

mixture was then loaded onto an Immobiline DryStrip (pH 3-10 NL, 7cm, GE Healthcare), which was rehydrated overnight in the dark and followed by isoelectric focusing (IEF) using a PROTEAN IEF system (Bio-Rad) according to the strip manufacturer's recommendations. Strips were reduced in equilibration buffer (6M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% dithiothreitol (DTT) for 20 min and thiols were then blocked with equilibration buffer containing 2.5% iodoacetamide for 20 min. Equilibrated strips were then applied to 12% polyacrylamide gels for SDS PAGE separation. Strips were embedded in molten agarose (0.5%) containing trace bromophenol blue atop 12% SDS PAGE gels, and electrophoresed at 90V for 30 min. followed by a constant voltage (120 V) using a mini PAGE system (Atto, Tokyo, Japan) until the dye front reached the end of the gel cassette. Gels were washed three times with Millipore water before scanning and colloidal coomassie staining(17). Four independent technical replicates of each 2DE gel were prepared. After electrophoresis, gels were scanned for fluorescence and then stained with colloidal coomassie as for 1DE.

Protein analysis and identification

Image analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics Limited, UK) to identify significantly altered spots in each treatment group in response to the drug. This software employs an alignment-based analysis approach which maps the same number of spots across all gels in a single analysis. The data obtained from protein gel images were tested for normality prior to any analysis and significance testing level was set at 0.05. Principal component analysis and analysis of variance (one way ANOVA) were performed for comparison and

assessment of statistically significant expression and redox variation between treatments and control. The spots were considered significantly different when $p < 0.05$ according to ANOVA and a fold change of > 1.5 were evident. Significant and well-resolved spots of sufficient intensity were selected for mass spectrometry (MS) analysis. These spots were manually excised using sterile pipette tips. Proteins were then digested in-gel with trypsin as previously described [7, 8]. Extracted peptides were loaded onto an R2 micro column (RP-C18 equivalent), desalted, concentrated and eluted directly onto a MALDI plate using α -cyano-4-hydroxycinnamic acid (5mg/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 analyser). The collected MS and MS/MS spectra were analysed in combined mode with the Mascot search engine (version 2.2; Matrix Science, Boston, MA) and the NCBI database restricted to 50 ppm peptide mass tolerance. No taxonomic 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 Service Unit, Instituto de Tecnologia Quimica e Biologica (ITQB), New University of Lisbon, Lisbon, Portugal.

Data analysis

Gel images for 1DE were analysed by Quantity One image analysis software (Bio-Rad, Hercules, CA, USA) measuring the total intensity for each lane. Fluorescence values were normalised for loading by dividing them with the colloidal coomassie staining intensity value for the same lane. Statistical analysis of enzymatic assay and

1DE were performed by one-way ANOVA. Homogeneity of variance was tested by Bartlett's method. Differences in variances and multiple comparisons were made by one-way analysis of variance followed by the Duncan *post hoc* test. All data are presented as means \pm SD.

Bioinformatics analyses

Hypothetical proteins were analysed by blastp to identify putative functionally-related proteins. Top-ranked related proteins of known function were analysed in the Pfam database to identify protein families/motifs [19] and appropriate gene ontology (GO) terms for each entry were identified in the UniProtKB database.

Results

All animals filtered water and fed normally during exposure. No animal morbidity or mortality was observed suggesting the animals were not extensively physiologically stressed by handling or exposure to diclofenac or solvent.

Biomarkers

GST activities were determined in extracts from both digestive glands and gills of animals exposed to 0.2 and 1 mg/L diclofenac as well as associated controls (Fig. 1; see also Supplementary Table 1). The levels of GST activity in mussel gills are typically 3-fold higher than in digestive gland [9]. In the present study there was no significant increase in gill or digestive gland GST activity in response to either dose of diclofenac used. By contrast, GR activities were increased by 100% in response to the 1mg/l dose of diclofenac (Fig. 2). Interestingly, GR is responsible for maintaining a sufficient supply of GSH which is generated by reducing GSSG and its elevation often forms part of an antioxidant stress response [20]. Interestingly,

there was a 50% decrease of GR in digestive gland at the 1mg/l dose, suggesting a tissue-specific difference in response. Catalase activity was unaffected in gill although it too was decreased by approximately 20% at the 1mg/l dose in digestive gland (Fig. 3).

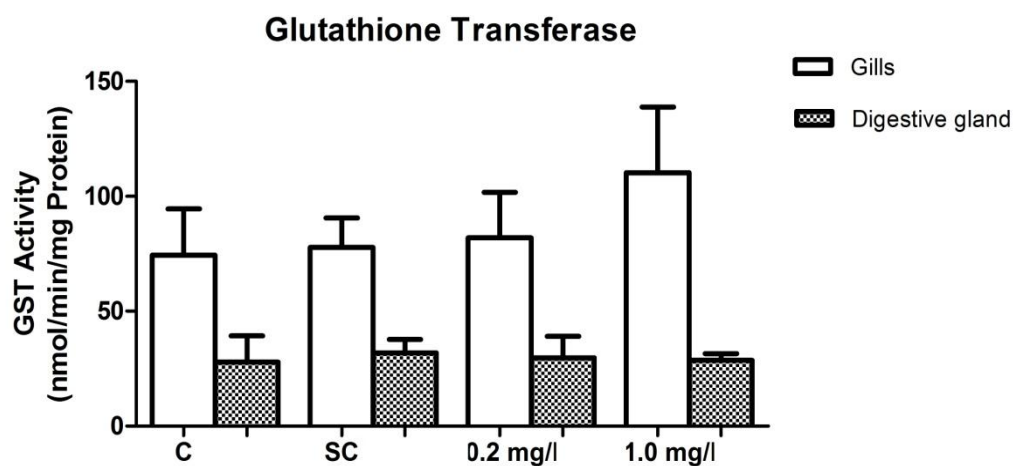


Figure 1. Glutathione transferase activity (GST; nmol CDNB conjugate form/min/mg protein) in gill and digestive gland of *M. edulis* exposed to diclofenac. Data are expressed as mean \pm SD. C: Control group, SC: Solvent Control group.

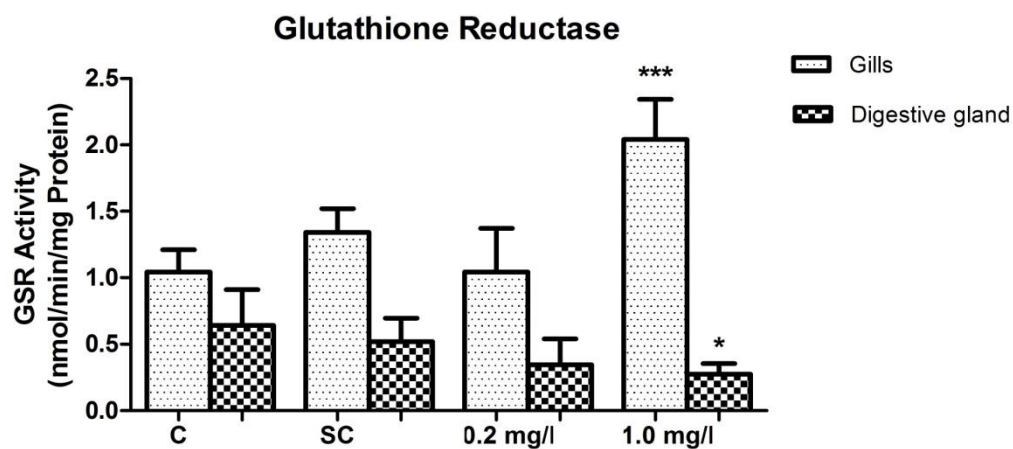


Figure 2. Glutathione reductase activity (GR; nmol CDNB conjugate form/min/mg protein) in gill and digestive gland of *M. edulis* exposed to diclofenac. Data are expressed as mean \pm SD. C: Control group, SC: Solvent Control group. Superscripts of different letters are significantly different from gill control group (C) and group 1.0 mg/l at *** p <0.001, gills solvent control group (SC) and group 1.0 mg/l at ** p <0.01, gills group 0.2 mg/l and group 1.0 mg/l at *** p <0.001 and digestive gland group control (C) and group 1.0 mg/l at * p <0.05.

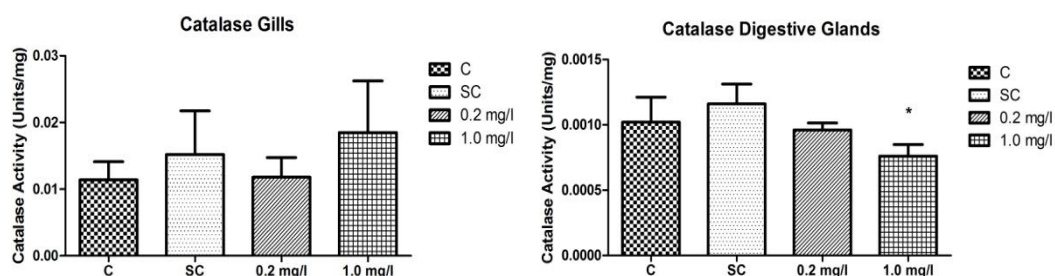


Figure 3. Catalase activity (CAT; U/min/mg protein) in gill and digestive gland of *M. edulis* exposed to diclofenac. Data are expressed as mean \pm SD. C: Control group, SC: Solvent Control group. Superscripts of different letters are significantly different from digestive gland control group and group 1.0 mg/l at * p <0.05

Proteomic analyses

Protein thiols were labelled with IAF and separated in 1DE. After scanning for fluorescence, gels were stained for protein with colloidal coomassie (see Supplementary material: Fig. S1 and Fig. S2A). Image analysis revealed that total protein thiol levels are consistently approximately three-fold higher in gill than digestive gland but that treatment with diclofenac reduced the gill levels markedly to

a level approximating those of digestive gland (Fig. 4). By contrast, levels of protein thiols in digestive gland remained unchanged at both doses of diclofenac used here. These observations suggest that protein thiols may be oxidised by exposure to ROS. It has been suggested that protein thiols are in fact key quantitative redox buffers in cells [21].

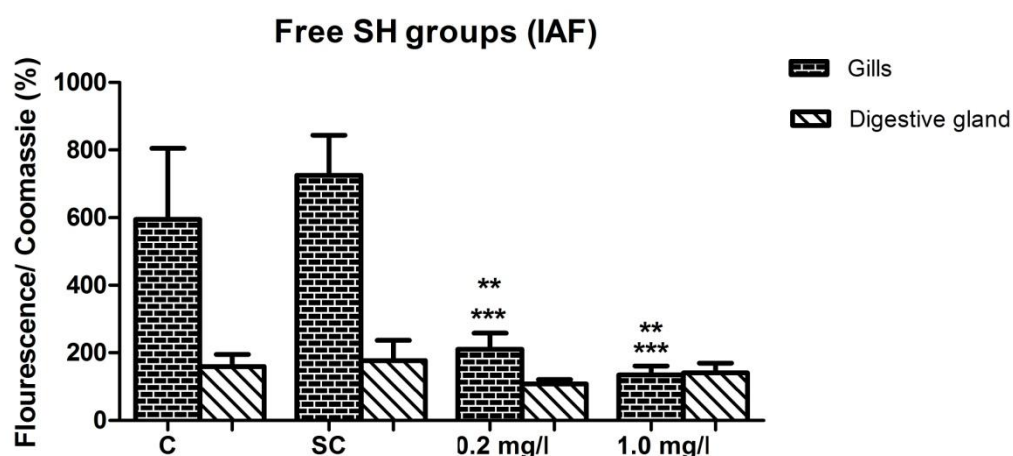


Figure 4. 1DE analysis of reduced thiols (IAF) content in gill of *M. edulis* exposed to diclofenac. Data are presented as average \pm SD (n=5). Superscripts of different letters are significantly different from gills, control group and group 0.2 mg/l **p<0.01, control group and group 1.0 mg/l **p<0.01, group solvent control and group 0.2 mg/l ***p<0.001, group solvent control and group 1.0 mg/l ***p<0.001.

To explore this further, total proteins for gills from the four treatment groups (control, solvent control, 0.2 mg/l and 1 mg/l) were separated in the pI range 3-10 by 2DE and both IAF fluorescence and coomassie-stained images were collected (Fig. 5; see Supplementary material: Fig, S2A). These showed well-separated single protein spots. Analysis with SameSpots software allowed selection of specific spots

for in-gel tryptic digestion. Zoom windows are illustrated for some spots in Fig 6. Spots were excised, digested with trypsin and identified by peptide mass fingerprinting with the aid of LC-tandem MS (Table 1). In all, 21 spots gave successful identifications representing 17 unique proteins of which six were hypothetical proteins. Because the genomes of mytilids are relatively poorly represented in sequence databases [22] only seven unique proteins were matched to entries from the genus *Mytilus* whilst ten were identified based on sequence similarity with other organisms. Four unique proteins were identified based on decrease in IAF fluorescence; caspase 3/7-4, heat-shock cognate protein 70, a predicted enolase-like protein and arginine kinase. Twelve unique proteins were identified based on changes in their abundance on exposure to diclofenac. Of these, five were hypothetical proteins, five were metabolic or antioxidant enzymes and two were structural proteins (Table 1). Arginine kinase was found in three separate spots, two of which (spots 310 and 311) were IAF labelled while the third (spot 542) showed change in abundance at the protein level.

Table 1. Identified protein spots using LC-MS/MS							
Spot ID	Protein	Accession 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	Fold change regulation
158 florescent	Caspase 3/7-4 [<i>Mytilus galloprovincialis</i>]	gi 325516449/ 22.83	-	-	6.4	-	↓; -1.4
196 florescent	Cytosolic heat shock cognate protein 70 [<i>Mytilus galloprovincialis</i>]	gi 77023195/ 17.55	404/100	376/100	7.7	8	↓; -1.5
237 florescent	PREDICTED: enolase-like [<i>Amphimedon queenslandica</i>]	gi 340368628/ 50.98	320/100	298/100	35	10	↓; -1.4
310 florescent	Arginine kinase [<i>Cuphodes sp. 6 ex Diospyros kaki/Diospyros japonica/Diospyros lotus</i>]	gi 299819008/ 20.43	101/99.721	101/99.998	7	1	↓; -1.3
311 florescent	Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	197/100	188/100	27	4	↓; -1.3
236	Predicted protein [<i>Hordeum vulgare subsp. vulgare</i>]	gi 326533846/ 72.70	92/97.93	88/99.866	30	4	↓; -1.5
299	Predicted protein [<i>Nematostella vectensis</i>]	gi 156403953/ 42.62	96/99.032	89/99.949	27	6	↓; -2.8
362	Beta-tubulin [<i>Mytilus edulis</i>]	gi 343455255 gb A EM36067.1/ 18.10	131/100	106/100	89	8	↓; -2.2
380	Hypothetical protein LOTGIDRAFT_206617 [<i>Lottia gigantea</i>]	gi 556103785/ 59.46	391/100	375/100	19	7	↓; -2.3
386	Actin [<i>Mytilus galloprovincialis</i>]	gi 5114428 gb AA D40314.1/ 41.72	57/98.221	50/99.985	31	7	↓; -1.6
482	Isocitrate dehydrogenase [<i>Mytilus galloprovincialis</i>]	gi 385268541 gb A FI56365.1/ 50.47	79/99.99	64/100	61	11	↑; +1.8
542	Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	197/100	188/100	27	4	↑; +2.0
544	RecName: Full=Heavy metal-binding protein HIP	gi 46395578 sp P8 3425.1 HIP_MYTED/ 24.27	147/100	131/100	59	7	↓; -1.7
548	RecName: Full=Heavy metal-binding protein HIP	gi 46395578 sp P8 3425.1 HIP_MYTE D/ 24.27	248/100	278/100	39	5	↓; -1.7
566	Mg:bb02e05 [<i>Xenopus laevis</i>]	gi 148223127/ 35.88	208/100	199/100	32	6	↓; -1.7
589	Cytosolic malate dehydrogenase [<i>Mytilus trossulus</i>]	gi 73656269 gb AA Z79366.1/ 36.37	72/99.949	65/99.999	27	5	↑; +1.5
610	PREDICTED: palmitoyl-protein thioesterase 1-like [<i>Musca domestica</i>]	gi 557774702/ 35.11	136/100	131/100	27	5	↑; +2.0
843	Proteasome subunit alpha type-2 [<i>Acromyrmex echinator</i>]	gi 332018472/ 25.88	101/99.721	84/99.813	42	6	↑; +1.5
1014	Hypothetical protein AND_06900 [<i>Anopheles darlingi</i>]	gi 312380904/ 17.83	114/99.986	83/99.806	55	6	↓; -1.9
1072	Superoxide dismutase [<i>Mytilus galloprovincialis</i>]	gi 215263232 emb CAQ68509.1/ 15.77	447/100	425/100	33	5	↑; +2.0
1212	Superoxide dismutase [<i>Mytilus chilensis</i>]	gi 332356353 gb A EE60900.1/ 15.76	340/100	311/100	50	7	↓; -1.9
^a The protein score probability limit (where p< 0.05) is 85. ^b Peptides with confidence interval above 95% were considered. ↑ Fold change increased ↓ Fold change decreased in related to control mussels							

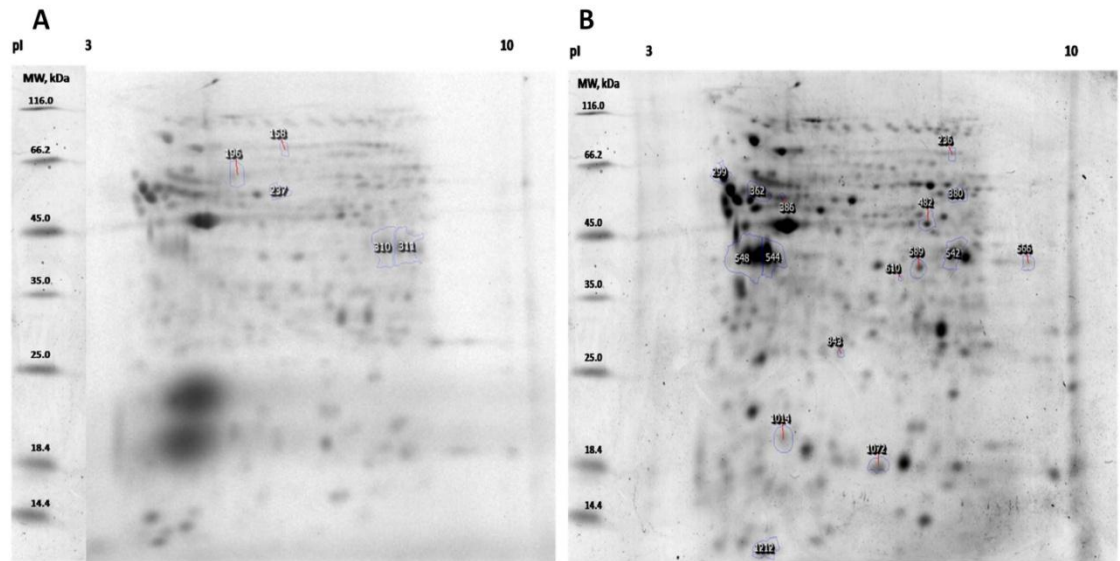


Figure 5. Annotated 2-DE image for gill extract of *M. edulis* exposed to diclofenac; Group treatment 1.0 mg/l, (A) Fluorescent IAF labelled (B) Coomassie staining with ID numbers identified by MALDI-TOF/TOF MS. The spots were selected by PCA and ANOVA ($p < 0.05$ and with a fold change > 1.5).

Bioinformatic analyses

A total of six proteins identified in this study (Table 1) were annotated as predicted or hypothetical proteins. None of these came from genus *Mytilus*. Hypothetical proteins are quite common in genomes [23] but are sometimes, as in this study, quantitatively implicated in stress responses [23, 24]. In order to gain insights into possible functions for these proteins, a blastp analysis was performed (Table 2). This revealed substantial sequence identity in the range 63-100% between the hypothetical proteins identified here and related proteins of known function. This would be regarded as predictive of very similar and probably identical structure and function. Three of the six proteins are most likely enzymes (alpha-enolase, ATP

synthase and palmitoyl-protein thioesterase), while a HSP-related stress-response, calcium-binding and initiation factor 5a protein were also identified. Family and motif analysis gave further insight to function with very low E-values and mapped closely to the sequences of the six hypothetical proteins listed in Table 1. GO analysis revealed evidence of stress-response in this group of proteins, notably with regard to protein folding and response to oxidative and metabolic stress. Two of the six proteins are most probably associated with the ER but one is associated with the lysosome and one with the mitochondrion. When combined with the other protein identifications these data are consistent with profound effects of diclofenac on a variety of key cellular processes including energy metabolism, the cytoskeleton, and stress response and protein turnover.

Table 2. Bioinformatic analysis of hypothetical proteins						
Spot ID	Accession number ^a	Best Hit ^a (Protein (<i>species</i>), E-value, % identity)	Pfam Family ^b (Residue range, E-value, Family:Clan)	Molecular Function ^c	Biological Process ^c	Cell Component ^c
236	gi 254540166	78 kDa glucose-regulated protein precursor (<i>Mus musculus</i>), E: 0.0 Identity: 83%	HSP70 31-636, 3.9 x 10 ⁻²⁷⁶ , PF00012:CL0108	ATP-binding [GO:0005524] Protein binding [GO:0005515] Mis-folded protein binding[GO:0051787] Ribosome binding [GO:0043024]	Protein unfolding response [GO:0006987] Response to ER stress [GO:0034976] Response to glucose starvation [GO:0042149]	ER lumen Cytoplasm Cell membrane
237	gi 229366696	Alpha-enolase (<i>Anoplopoma fimbria</i>), E:0.0 Identity: 100%	Enolase_N 3-134, E:1.7 x 10 ⁻⁵⁹ , PF03952:CL0227 Enolase_C 143-431, 2.7 x 10 ⁻¹⁶² , PF00113: CL0256	Magnesium ion binding [GO:0000287]	Glycolytic process [GO:0006096]	Phosphoenol pyruvate complex
299	gi 150404776	Calreticulin (<i>Pinctada fucata</i>), E:0.0 Identity: 80%	Calreticulin 20-331, 5.4 x 10 ⁻¹²⁴ , PF00262: na ^d	Calcium ion binding [GO:0005509]	Protein folding [GO:0006457]	ER
380	gi 116008297	Mitochondrial H ⁺ ATPase, a subunit (<i>Pinctada fucata</i>), E:0.0 Identity: 86%	ATP-synt_ab_N 69-135, 1.1 x 10 ⁻¹⁸ , PF02874:CL0275 ATP-synt_ab 191-415, 6.3 x 10 ⁻⁷¹ , PF0006:CL0023 ATP-synt_ab_C 427-517, 6.5 x 10 ⁻²⁴ , PF00306:na ^d	ATP-binding [GO:0005524] ATP-synthase activity [GO:0046933]	ATP-hydrolysis coupled proton transport [GO0015991]	Proton-transporting ATP Synthase complex
610	gi 24640652	Palmitoyl-protein thioesterase 1 isoform a (<i>Drosophila melanogaster</i>), E:1 x 10 ⁻¹²⁸ Identity: 63%	Palm_thioest 38-311, E: 2.7 x 10 ⁻¹⁰⁴ PF02089:CL0028	Palmitoyl-(protein) hydrolase activity [GO:0008474]	Determination of adult life-span [GO:0008340] Protein depalmitoylation [GO: 0002084]	Lysosome
1014	gi 568259069	Initiation factor 5a (<i>Anopheles darlingi</i>), E:1x 10 ⁻⁹⁵ Identity: 99%	KOW 29-59, E:0.0002 PF00467:CF0107 eIF5a 85-152, E:9.2 x 10 ⁻²⁶ PF01287:CF0021	Ribosome-binding [GO:0043022] Translation IF activity [GO:0003743]	Peptidyl-lysine modification to peptidyl-hyusine [GO 0008612] Positive regulation of translation termination [GO:0045905]	None predicted
^a Best hit results obtained using a blastp search. ^b Families and GO functions obtained from Pfam. ^c Molecular function, biological process and cell components were obtained from UniProtKB. ^d No clan listed						

Discussion

The animals used in this study were physically healthy and were observed to filter water normally during the experiments suggesting they did not experience significant physiological stress.

Biomarkers

Antioxidant enzymes represent a key defence mechanism to enable invertebrates to protect themselves against oxidative stress [6]. GST activities showed no significant difference in gills and digestive glands for both diclofenac doses when exposed for 7 days. This may be attributable to the relatively short exposure time since 21 days diclofenac exposure significantly reduced GST activity in digestive gland [7]. GSTs' main roles in detoxification involve either catalysing GSH conjugation or ligand binding and gill has significantly more GST activity than other mussel tissues [9]. It is possible there is sufficient GST capacity in gill already to cope with the doses of diclofenac used. No significant change was observed in CAT activity of gill although a 20% decrease was observed digestive gland. GR activities were 100% increased in gills and 20% significantly decreased in digestive gland in response to the highest diclofenac dose. These findings agree with previous observations that diclofenac and other pharmaceuticals are capable of modifying the antioxidant enzyme defence profiles of mussel tissues [6, 7, 25]. The 100% significantly increase in GR in gill is likely to support antioxidant defence as this is the key enzyme responsible for maintaining sufficient GSH to withstand oxidative stress [13, 26].

Proteomic and Bioinformatics analyses

The findings discussed in the previous paragraph made it seem likely to us that the gill proteome might be affected by diclofenac as we have previously found for digestive gland [7]. Significant changes in abundance were observed for approximately 30 proteins of which 21 spots yielded successful identifications. The protein abundance changes were moderate (mostly less than 2.8 fold) whilst decreases in fluorescence were even more modest (Table 1). This suggests that the gill proteome is resilient to the effects of diclofenac under the conditions used in this study. Four unique proteins were identified based on 1.3-1.5-fold decrease in IAF fluorescence; caspase 3/7-4 (id: 158), cytosolic heat-shock cognate protein 70 (id: 196), a predicted enolase-like protein (id: 237) and arginine kinase (id: 310 and 311). This decrease is interpreted as evidence for thiol oxidation in the case of these four proteins. Caspases are a gene family which are important for sustaining homeostasis through regulating apoptosis and inflammation. Therefore, Caspase-4 plays important roles in human inflammation [27]. In *M. galloprovincialis*, caspase genes' tissue expression patterns revealed extremely high expression levels within gill, where the apoptotic process is highly active due to the clearance of damaged cells. Apoptosis is likely to be tightly regulated in bivalve molluscs by over-expression or suppression of caspase genes. There is previous evidence for caspase-specific responses to pathogens and pollutants [28]. Heat-shock proteins (HSPs) are well-known stress biomarkers because of their synthesis in response to stress conditions [29]. Several studies have been performed on HSP 70 expression in marine organisms; pacific oyster (*Crassostrea gigas*), blue mussels (*M. edulis*), Mediterranean mussels (*M. galloprovincialis*) and clam (*Ruditapes decussates*) which found decreased levels of HSP 70 in gills in response to toxicants [30-33].

Oxidation of cytosolic heat-shock cognate protein 70, identified in the present study, suggested that diclofenac induced cellular stress. During such stress conditions, HSPs move into the cell nucleus and start to repair and protect nuclear proteins from aggregation which would lead to cell damage [34]. Enolase, also known as phosphopyruvate hydratase, is an abundantly expressed cytosolic protein which is widespread in many tissues [35]. Alpha-Enolase is considered as a marker of pathological stress in disease [35] and it has also been described as a neurotrophic factor, a HSP (HSP48) and as a hypoxic stress protein [36]. Arginine kinase belongs to the phosphagen kinase family that catalyzes the reversible transfer of a high-energy phosphate from the phosphagen arginine phosphate to ADP to form ATP [37]. Arginine kinase functions in protein turnover. In muscles of marine organisms, activities of ATP turnover occur in phasic adductor of the scallop and the abdominal flexor muscles of the lobster [38]. Interestingly, of the four proteins in which thiols seem to have been oxidised, only arginine kinase showed also a change in abundance. The significance of this is discussed in more detail in the penultimate paragraph below.

Five other hypothetical proteins were also identified here. 78 kDa glucose-regulated protein precursors (id: 236) is a member of the HSP70 family ranging in size from 66 kDa to 78 kDa. This is one of the best-studied stress-response protein families [39]. A glucose-regulated HSP70 (grp78) is localised in the lumen of the endoplasmic reticulum (ER) where it binds to incompletely assembled multimeric proteins and to underglycosylated or denatured proteins [40]. The grp78 gene is expressed at high levels in secretory cells, such as hepatocytes, and its expression is further increased when protein processing is blocked in the ER [41]. Calreticulin (id: 299) has two principal functions which are involved in maintaining calcium in

organisms and acting as a chaperone to aid in protein folding [42]. This protein has been reported to be up-regulated and involved in protein refolding and the ER stress response [43]. Mitochondrial H⁺ ATPase, a subunit (id: 380) is a component of one of the cell's most important enzymes. This comprises two rotary motors which work together to interconvert energy. Dysfunction in this enzyme may lead to loss of efficient energy transduction [44]. Palmitoyl-protein thioesterase 1 isoform (id: 610) is involved in protein palmitoylation, a common lipid-based modification. It is known to regulate diverse aspects of neuronal protein trafficking and function. Several emerging roles have been suggested in various aspects of pathophysiology for protein palmitoylation because of its potential to influence protein transport between intracellular compartments [45]. Initiation factor 5a (id: 1014) plays a very important role in cell proliferation and senescence as a biological switch in dividing and dying cells [46].

Other proteins identified in this study were beta tubulin, actin, isocitrate dehydrogenase, cytosolic malate dehydrogenase, proteasome subunit alpha type-2, superoxide dismutase and heavy metal-binding protein HIP. Beta tubulin (id: 362), one of the most representative mussel adhesion and mortality-related transcripts previously identified [47], showed a decrease in abundance. It was previously reported in *M. edulis*, as forming disulphide bonds in response to menadione [48]. Actin (id: 386), which also decreased in abundance is a known target in *M. edulis* for glutathionylation and carbonylation under oxidative stress conditions [49]. In relation to diclofenac exposure to zebrafish (*Danio rerio*), it was previously shown that actin organization and muscle fiber alignment are disturbed thereby inducing a malformed somite phenotype [50]. Isocitrate dehydrogenase (id: 482) as part of the Krebs cycle, is very involved in energy metabolism and significantly decreased in

abundance here. It has previously been suggested to play a role in antioxidant defence [51]. The repair process for oxidative damage depends heavily on NADPH levels in the cell as these reducing equivalents are necessary both for GR and thioredoxin reductase which help to maintain cellular redox status [52, 53]. In the present study, cytosolic malate dehydrogenase (id:589) increased in protein abundance across the exposure group. This protein is a widely-distributed and essential enzyme that catalyzes the interconversion of malate to oxaloacetate with reduction of NAD or oxidation of NADH [54]. Previous studies found decreased labeling with IAF in cytosolic malate dehydrogenase in digestive glands of *R. decussatus* (Mediterranean clams), suggesting oxidative stress induced in response to permethrin, a type I pyrethroid insecticide exposure [55]. Proteasome subunit alpha type-2 (id: 843) also decreased in protein abundance which agreed with previous work in which proteasome subunit alpha type-2 was significantly altered in male mussel gills exposed to BDE 47, a brominated flame-retardant [56]. Superoxide dismutase (SOD; id: 1072 and 1212) showed increased in protein abundance for both spots. Superoxide dismutase (SOD) is one of the most important antioxidant defence enzymes in aerobic organisms [57]. SOD activity increased in liver and gills of *Cyprinus carpio* (common carp) after 72h exposure to diclofenac [58]. Oxidative metabolism of diclofenac favours formation of O² anion which triggers an increase in this biomarker [58]. Two spots were also identified as containing heavy metal-binding protein HIP (id: 544 and 548). These both decreased in protein abundance on exposure to diclofenac. Huntingtin interacting protein (HIP) protein was previously isolated from hemolymph of *M. edulis* and binds heavy metals ions (Cd, Cu and Zn) thus contributing to their detoxification [59, 60].

Some aspects of the abundance and oxidation changes observed are intriguing and suggest a complicated and subtle change in protein expression signature in response to diclofenac. For example, arginine kinase was found to be oxidised in two protein spots whilst its abundance increased twofold in spot 542 (Table 2). This suggests that the oxidation effect must be especially significant in the case of this protein as there is more of it and it is increasingly oxidised. Arginine kinase plays a key role in maintaining high levels of phosphagens of which phosphoarginine is most important in invertebrates (functionally equivalent to phosphocreatine in vertebrates) [61]. Phosphagens allow rapid replenishment of ATP which suggests diclofenac may be affecting energy metabolism. It is known that a scallop arginine kinase has a high binding affinity for actin [62]. This may suggest scope for interplay between energy metabolism and the cytoskeleton in which both actin and tubulin of mussels (both identified here as decreasing in abundance) have been shown to be redox-sensitive [48, 49]. The dehydrogenases, isocitrate dehydrogenase and cytosolic malate dehydrogenase, both increased in abundance again suggesting a possible effect of diclofenac on cellular energy metabolism. Lastly, there is a curious effect on SOD in which its abundance increases two-fold in one spot (spot 1072) whilst decreasing in another (spot 1212) (Table 2; Fig. 6). It is noteworthy that the latter spot has a smaller mass and more acidic pI than the latter which suggests that spot 1212 may represent a proteolytic product of spot 1072. This change in relative abundances would be consistent with increased production of superoxide dismutase, a key component of antioxidant defence.

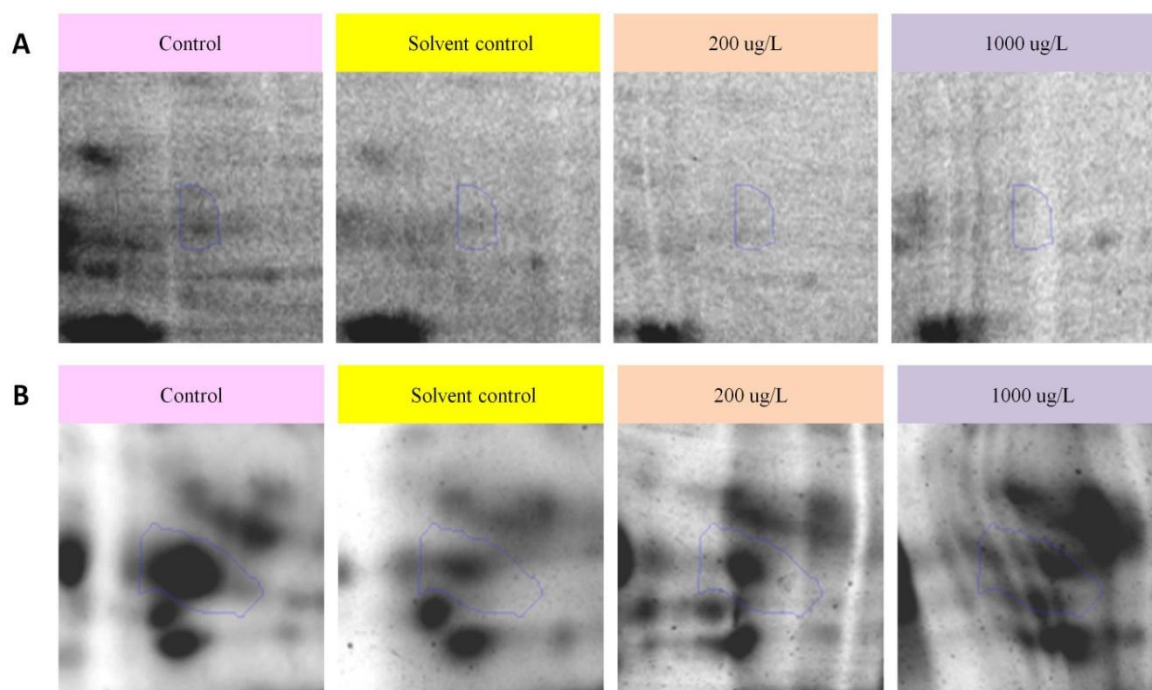


Figure 6. Representative 2-DE image of gill extract from *M. edulis* exposed to diclofenac. The spots were identified by MALDI-TOF/TOF MS. **(A)** Fluorescent IAF labelled spot (id: 196) identified as cytosolic heat shock cognate protein 70 [*M. galloprovincialis*]/ gi|77023195 and **(B)** Coomassie staining of the spots (id: 362) identified as beta-tubulin [*M. edulis*]/ gi|343455255|gb|AEM36067.1|

In conclusion, after exposure of *M. edulis* to diclofenac for 7 days, gills show effects on antioxidant enzyme activity profile and a striking decrease in total protein thiols. A differential protein expression signature was identified in gills suggesting diclofenac has profound effects on proteins involved in energy metabolism, the cytoskeleton, stress-response and protein modification, transport and turnover. In addition, four unique proteins were found to be directly oxidised. This study extends previous work demonstrating that redox proteomics is a robust tool to investigate stress response of pharmaceuticals on non-target organisms in the aquatic

environment. We argue that environmental exposure of this non-model organism to diclofenac is likely to compromise their long-term viability.

Acknowledgements

Siti NurTahirah Jaafar received an Academic Training Scheme scholarship from Malaysian Government and Universiti Malaysia Terengganu. The authors thank Dr Maria O'Mahony, manager of the AFDC (Aquaculture and Fisheries Development Centre), School of Biological, Earth and Environmental Sciences, University College Cork, Ireland for facilitate the facility for exposure experimental work.

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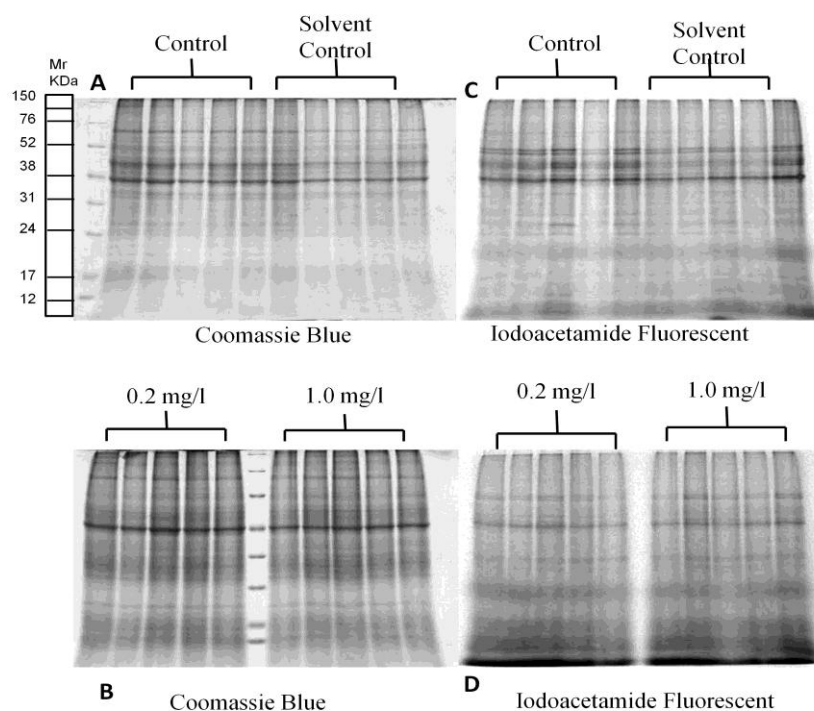
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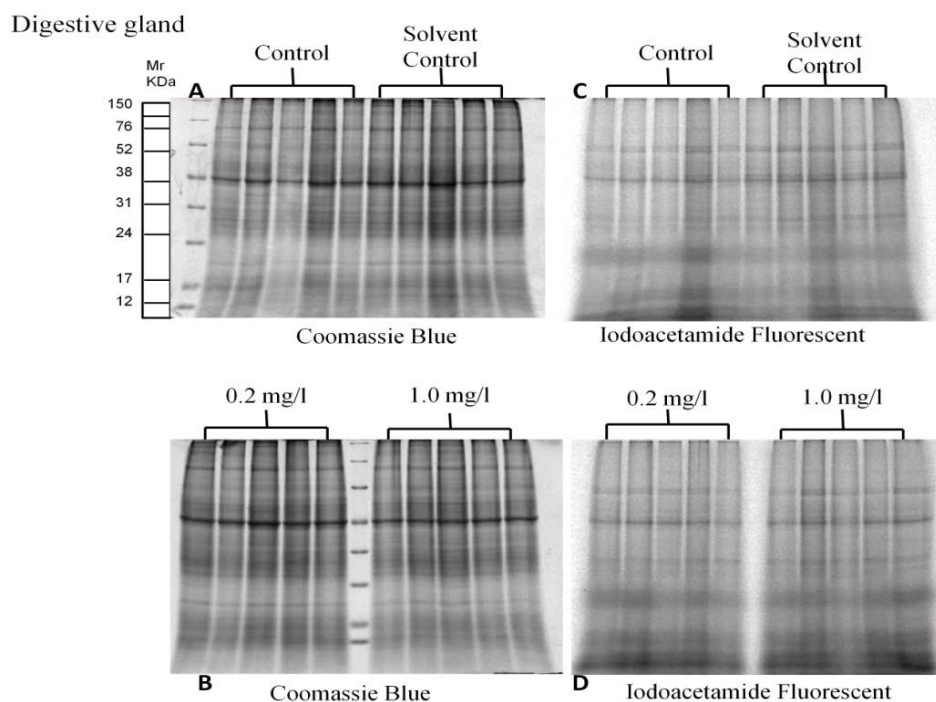
Supplementary materials.

Supplemental Table 1. Enzyme activity measurements (min \pm SD) from <i>M. edulis</i> gill and digestive gland. The groups are control (C), solvent control (SC), 0.2 mg/l and 1.0 mg/l diclofenac.				
Enzyme	C	SC	0.2 mg/l	1.0 mg/l
(nmol/min/mg)				
GST				
Gill	74.476 \pm 20.034	77.802 \pm 12.794	81.892 \pm 19.751	110.316 \pm 28.452
Digestive gland	27.858 \pm 11.460	31.802 \pm 5.826	29.702 \pm 9.342	28.578 \pm 3.023
(nmol/min/mg)				
GR				
Gill	1.043 \pm 0.1679	1.343 \pm 0.176	1.042 \pm 0.328	2.040 \pm 0.302***
Digestive gland	0.639 \pm 0.271	0.520 \pm 0.175	0.343 \pm 0.197	0.273 \pm 0.079*
(U/mg)				
Catalase				
Gill	0.0114 \pm 0.0027	0.0152 \pm 0.0065	0.0118 \pm 0.00295	0.0185 \pm 0.00774
Digestive gland	0.00102 \pm 0.00019	0.00116 \pm 0.000152	0.00076 \pm 5.47723E-05	0.00076 \pm 8.94E-05*
* p < 0.05				
*** p < 0.001				

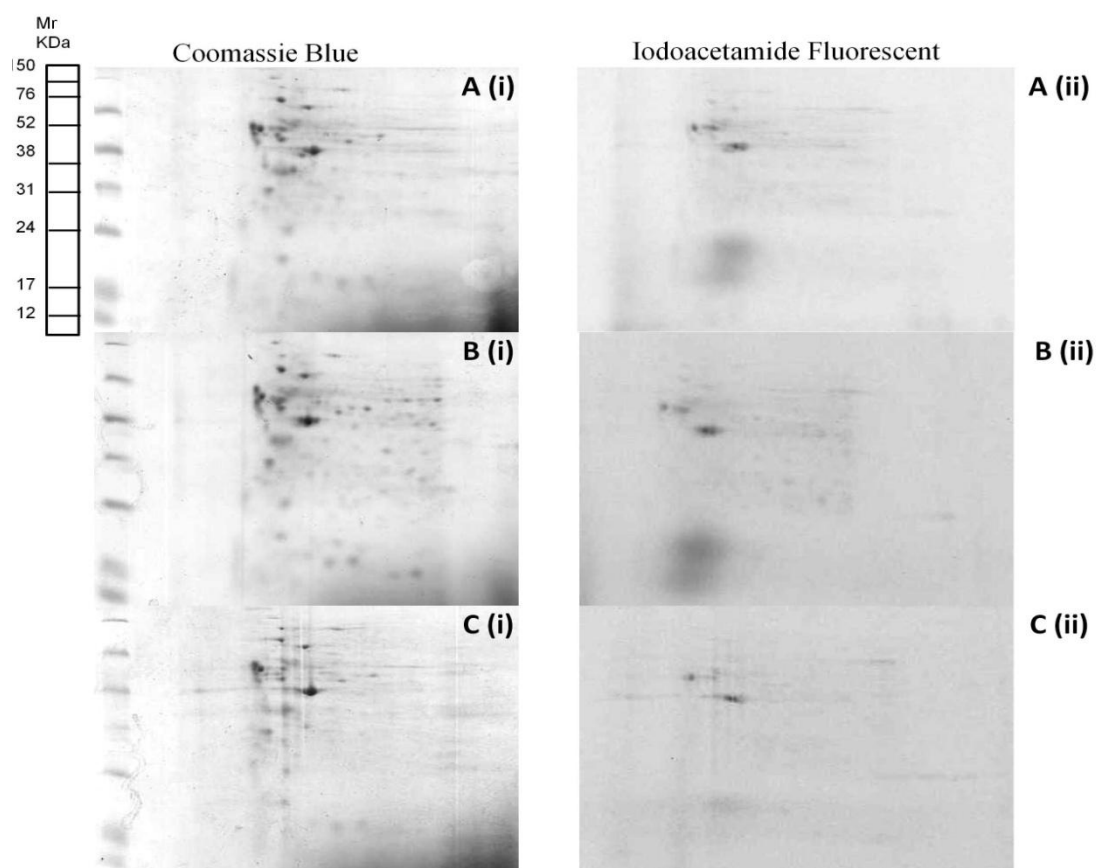
Gill



S1. Representative images of 1DE SDS-PAGE of gill cytosolic protein extracts from *M. edulis* exposed to diclofenac. Visualization of total protein levels by colloidal coomassie staining of gill (A) and (B). Fluorescence images of free thiol-containing proteins labelled with 5-iodoacetamidofluorescein (IAF) in gill (C) and (D). Gels contain molecular weight markers (Mr) and 25µg of protein from each lane was loaded for 5 biological replicates each Group: Control, Solvent Control, 0.2 mg/l and 1.0 mg/l.



S2(A). Representative images of 1DE SDS-PAGE of digestive gland cytosolic protein extracts from *M. edulis* exposed to diclofenac. Visualization of total protein levels by colloidal coomassie staining of digestive gland (A) and (B). Fluorescence images of free thiol-containing proteins labelled with 5-iodoacetamidofluorescein (IAF) in digestive gland (C) and (D). Gels contain molecular weight markers (Mr) and 25µg of protein from each lane was loaded for 5 biological replicates each Group: Control, Solvent Control, 0.2 mg/l and 1.0 mg/l.



S2 (B). Annotated 2-DE image for gill extract of *M. edulis* exposed to diclofenac; Control group; Coomassie staining [A(i)] Fluorescent IAF labelled [A(ii)]; Solvent control group; Coomassie staining [B(i)] Fluorescent IAF labelled [B(ii)] and group treatment 0.2 mg/l; Coomassie staining [C(i)] Fluorescent IAF labelled [C(ii)].

CHAPTER 4:

(Redox proteomic analysis of *Mytilus edulis* gills: Toxic effects of the pro-oxidant Copper)

Submitted to Proteomics

Redox proteomic analysis of *Mytilus edulis* gills: Toxic effects of the pro-oxidant Copper

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Keywords:

Copper; proteomics; mytilus; redox; toxicology; ecotoxicology

Abstract

Environmental copper is a key source of contamination in marine ecosystems. In this study, the blue mussel, *Mytilus edulis*, was exposed to sub-lethal doses of copper (50 µg/L, 150 µg/L and 300 µg/L) for 1 hour with 24 hours recovery time. Effects on the gill, the principal feeding organ of mussels, were investigated, followed by measurement of enzymatic activities, and protein thiol status. Oxidation of protein thiols - detected by tagging thiols with 5-iodoacetamido fluorescein (IAF) -was studied using one-dimensional electrophoresis and two-dimensional electrophoresis (2DE). It was noted that there were significant differences in gill glutathione transferase activities, whilst no effect was evident on gill glutathione reductase or catalase activities. Gill total protein thiols increased in response to 150 µg/L copper before decreasing significantly at 300 µg/L. In contrast, digestive gland protein thiols were maximal at 300 µg/L. Protein 2DE profiles were compared with Progenesis SameSpots software both for IAF fluorescence and protein abundance. These were matched with a protein dataset previously obtained in response to diclofenac (Jaafar et al., Redox proteomic analysis of *Mytilus edulis* gills: Effects of the pharmaceutical diclofenac on a non-target organism. *Drug Test. Anal.* 2015. IN PRESS). This revealed five proteins common to both 2DE profiles. Three proteins responded similarly to diclofenac and copper (beta tubulin, arginine kinase, superoxide dismutase) whilst two proteins responded differently (IAF-calreticulin, heat shock cognate protein 70). Both of the latter increased in response to copper but decreased in response to diclofenac. This supports redox proteomics as a strategy capable of detecting common responses to pro-oxidants but also versatile enough to reveal subtle differences between their effects.

Introduction

Heavy metals are very common ecosystem pollutants especially in the marine environment. Copper is ubiquitous in the Earth's crust and it is often an anthropogenic pollutant in marine ecosystems [1]. In most organisms it is imbibed orally and copper is consistently one of the most toxic dietary metals, especially to aquatic organisms [2, 3]. Copper is an essential micronutrient and copper ions are required for electron-transfer reactions in respiration, free radical defence, neuronal myelination, angiogenesis, and other processes [4, 5]. It is a key component of metalloenzymes such as superoxide dismutases, laccases and oxidases where it is involved in accepting or donating electrons by redox cycling between the Cu I and Cu II oxidation states [4, 6]. It also frequently contributes to maintenance of protein structure and in DNA binding through its coordination chemistry [7]. Free unbound copper ions can interact with molecular oxygen, generating reactive oxygen species (ROS) and causing subsequent oxidative damage *via* Haber/Weiss and/or Fenton reactions [8]. ROS produced by copper include the hydroxyl ($\text{HO}\cdot$) and superoxide (O_2^-) free radicals and hydrogen peroxide (H_2O_2) [9]. Overproduction of ROS can cause substantial stress/oxidative damage to biomolecules, (lipids, proteins, DNA), eventually leading to cellular toxicity [10].

Bivalves are excellent sentinel organisms for the study of heavy metal toxicity in aquatic ecosystems [11]. The blue mussel, *Mytilus edulis*, and other genus *Mytilus* species, are the organisms used most extensively for aquatic toxicology research worldwide [12, 13]. They are abundant, euryhaline, sedentary organisms with high filtration rates, easy handling, and widespread geographical distribution [13, 14]. Other molluscs used for the study of toxic effects of heavy metals include clams [15] and oysters [16].

There has been interest in recent years in exploiting proteomics approaches to study ecotoxicology in more molecular detail but, surprisingly, this has not been extensively applied to studies with *Mytilus* [14, 17-19]. In a landmark study, Shepard et al., identified a protein expression signature (PES) from a whole-animal extract of *M. edulis* exposed to copper [20]. However, these workers did not obtain specific protein identifications nor could this PES be attributed to any particular mussel tissue. More recently, Maria et al. 2013 used a proteomic approach to identify 18 proteins in a study of gills from *M. galloprovincialis* exposed to copper and benzo (a) pyrene or both [21]. This revealed effects on glutathione transferase (GST) and proteins involved in digestion, growth and remodelling processes in response to copper treatment. We have explored the idea of using “redox” proteomics whereby, as well as detecting effects on protein abundance, information on protein redox status can also be obtained [22]. The aim of the present study was to determine toxic effects of pro-oxidant copper using a redox proteomic approach. This involved the study of a complex PES and protein thiol redox status using one-dimensional and two-dimensional electrophoresis (1DE, 2DE) in gills of *M. edulis*. In addition, antioxidant enzyme activities were measured in gills and digestive glands of mussels exposed to known doses of copper.

Materials and methods

Chemicals

Copper chloride, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), EDTA, phenyl-methylsulphonylfluoride (PMSF), reduced glutathione (GSH), oxidised glutathione (GSSG), 5-(iodoacetamido) fluorescein (IAF) and copper chloride were purchased from Sigma Aldrich. All other reagents used were of analytical grade.

Animals and sample preparation

One hundred mussels, (*M. edulis*, 5-6 cm in length), were collected from a clean site (9° 30'W, 51°40'N) in Bantry Bay, County Cork, Ireland in the middle of March 2013. This sampling location area is well-known for commercial mussel aquaculture [23]. Animals were collected from the intertidal zone at low tide and transported to the laboratory. They were cleaned and acclimated for a week in artificial sea water (ASW) at $10^{\circ} \pm 1^{\circ} \text{C}$ and the salinity was 32ppt with a 12-h light/dark cycle. Mussels were fed daily with a commercial solution (Shellfish diet 1800) at the recommended 2% maximum feed conversion rate during acclimation and exposure. The animals were exposed to three concentrations of copper chloride (50 µg/L, 150 µg/L and 300 µg/L, respectively) with daily water change. Copper chloride was dissolved in distilled water and added to the tanks after water change. Four groups were established for the experiment: control mussels (ASW only); 50µg/L (ASW+dH₂O+ copper chloride); 150 µg/L (ASW+dH₂O+ copper chloride); 300 µg/l (ASW+dH₂O+ copper chloride). Each of the groups contained 5 biological replicates, each consisting of 5 pooled animals. The mussels were fed for 4 hours prior to exposure. Water was changed and the mussels were then exposed to copper for an hour.

Mussels were then allowed to recover for 24 hours before tissue dissection. No mortality was observed under the conditions used and all animals were observed to be feeding normally. Gill and digestive gland were dissected on ice and pooled in groups from five individual animals. The samples were homogenized in a motor-driven Teflon Potter-Elvehjem homogenizer in Tris-HCl buffer containing 10 mM Tris-HCl, 0.5 M sucrose, 0.15 M KCl, 1mM EDTA and 1mM PMSF, pH 7.2, at a weight: volume ratio of 1:3. The homogenate was centrifuged at 15 000 g for 1h at 4°C and the supernatant was collected and stored at -80°C for further analysis. The protein content was estimated by the Bradford method using BSA as a standard [24].

Antioxidant activity

GST activity was quantified in gill and digestive gland tissues from sample aliquots containing 15 µg of protein diluted to a volume of 50 µl. Samples were loaded into a 96-well microtitre plate with 100 µl of 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) (from a 40 mM stock in ethanol) in 0.15 M potassium phosphate buffer (pH 6.5) [25, 26]. GST activity was measured spectrophotometrically by adding 50 µl of 20 mM GSH and measuring absorbance at 340nm immediately and every 15 s for 5 min. The specific activity was expressed as nMol CDNB conjugate formed/min/mg protein; $\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Glutathione reductase (GR) activity was determined using β -NADPH as substrate [27]. The reaction rate was detected at 340nm and specific activity was expressed as nMol NADPH consumed/minute/mg protein; $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. Catalase activity (CAT) was measured as the decrease in 240 nm absorbance due to consumption of hydrogen peroxide (H_2O_2) [28]. Specific activity was expressed as U/min/mg protein; $\epsilon = 0.04 \text{ mM}^{-1} \text{ cm}^{-1}$.

Iodoacetamido fluorescein (IAF) labelling

Protein thiols were labelled with 0.2 mM IAF from a 20 mM stock solution in dimethyl sulphoxide [29]. Gill sample aliquots containing 150 µg protein (1DE) and 300 µg protein (2DE) were incubated with IAF for two hours on ice in the dark. Proteins were precipitated by incubating extracts in 10% trichloroacetic acid (TCA) for 5 min on ice, followed by centrifugation at 11,000 g for 3 min. The resulting pellet was washed in an excess of ice-cold acetone to remove TCA and any interfering salts or non-protein contaminants. Protein extracts were re-suspended in 75 µl sample buffer for one-dimensional electrophoresis (1DE; 62.5 mM Tris HCl, pH 6.8) containing 25% glycerol, 2% SDS, 5%, β-mercaptoethanol and a trace amount of bromophenol blue) or 125 µl rehydration buffer for 2DE (7 M urea, 2 M thiourea, 2% CHAPS, 4% ampholyte (Pharmalyte 3-10 pH), 1.2% DeStreak reagent and a trace amount of bromophenol blue). IAF reacts only with reduced thiols (-SH) but not with oxidised variants such as sulphenic acids (-SOH) or disulphides (-S-S) which might be expected to form on oxidative stress [29].

One-Dimensional electrophoresis

1DE was performed in 12% polyacrylamide gels [30]. Fluorescently-labelled proteins (30 µg per lane) were loaded on four replicate lanes per sample. All proteins were dissolved in sample buffer (dH₂O; 0.5 M Tris HCl, pH 6.8; glycerol; 10% SDS; 0.5% bromophenol blue), and electrophoresed (4°C) at 120V for two hours in running buffer (10X stock: 30.0g Tris base; 144.0g glycine; 10.0g SDS; 1L H₂O). Following electrophoresis, gels were scanned in a Typhoon scanner, model 9410 (Amersham Biosciences) with excitation wavelength set at 488 nm and emission

wavelength at 520 ± 20 nm filter. Gels were then stained with colloidal coomassie followed by destaining [31]. Gels were then scanned in a calibrated densitometer (BioRad, Hercules, CA, USA).

Two-dimensional electrophoresis

Homogenates (300 μ g protein) were labelled with IAF as stated in the previous section. Protein pellets (150 μ g protein) were resuspended in 125 μ l rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 1.2% bis (2-hydroxyethyl)-disulfide, 4% ampholytes (3-10 for IEF, GE Healthcare) and a trace amount of bromophenol blue [28, 29]. The protein mixture was loaded onto an Immobiline DryStrip (pH 3-10 NL, 7cm, GE Healthcare), which was rehydrated overnight in the dark followed by isoelectric focusing (IEF) using a PROTEAN IEF system (Bio-Rad) according to the strip manufacturer's recommendations. After focusing, strips were reduced in equilibration buffer (6M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% DTT for 20 min and thiols were blocked with equilibration buffer containing 2.5% iodoacetamide for 20 min. The equilibrated strips were embedded in molten agarose (0.5%) containing trace bromophenol blue atop 12% SDS PAGE gels, and electrophoresed at 90V for 30 min. followed by a constant voltage (120 V) using a mini PAGE system (Atto, Tokyo, Japan) until the dye front reached the end of the gel cassette. Gels were washed three times with Millipore water before scanning and colloidal coomassie staining [31]. Four independent technical replicates of each 2DE gel were prepared. After electrophoresis, gels were scanned for fluorescence and then stained with colloidal coomassie as for 1DE. Image analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics

Limited, UK) to identify significantly altered spots in each treatment group in response to copper. This software employs an alignment-based analysis approach which maps the same number of spots across all gels in a single analysis. The data obtained from protein gel images were tested for normality prior to any analysis and significance testing level was set at 0.05. Principal component analysis and analysis of variance (one way ANOVA) were performed for comparison and assessment of statistically-significant expression and redox variation between treatments and control. The spots were considered significantly different when $p < 0.05$ according to ANOVA and a fold change of > 1.5 were evident.

Protein identification

The 2DE images obtained in this study were matched with SameSpots software with 2DE separations obtained in our laboratory in a previous study of *M. edulis* exposure to diclofenac [32]. This allowed rapid discovery of proteins previously-identified by mass spectrometry. In addition, spots of interest were also manually compared to a reference proteome for *M. edulis* [33].

Data analysis

Background reduction and optical density quantification of protein bands in 1DE gels was performed using Quantity One image analysis software (Bio-Rad). The intensity of fluorescence values were normalised against protein content measured for the same electrophoresis lane. This measurement was performed to correct for slight differences in sample loading and enabled the extent of thiol oxidation to be compared between samples. Statistical analysis of enzymatic assay and 1DE were

performed by one-way ANOVA. Homogeneity of variance was tested by Bartlett's method. Differences in variances and multiple comparisons were made by one-way analysis of variance followed by the Duncan *post hoc* test. All data are presented as means \pm SD.

Results and discussion

Antioxidant activity

As well as being a target for thiol oxidation *via* disulphide bridge formation in oxidative stress in *M. edulis* [14, 17], GSTs directly detoxify oxidation products such as peroxides formed as a consequence of oxidative stress [34]. GST activities were determined in extracts from both gill and digestive gland of animals exposed to 50, 150 and 300 µg/L copper chloride as well as associated controls (Fig. 1). There was a significant increase in gill GST activity in response to 50 and 300 µg/L copper chloride. However, no significant differences were observed for GST activity in digestive gland or for 150 µg/L, in the case of gill. In a study in *M. galloprovincialis* gill, a two-fold GST induction has previously been reported based on abundance in 2DE gels [21].

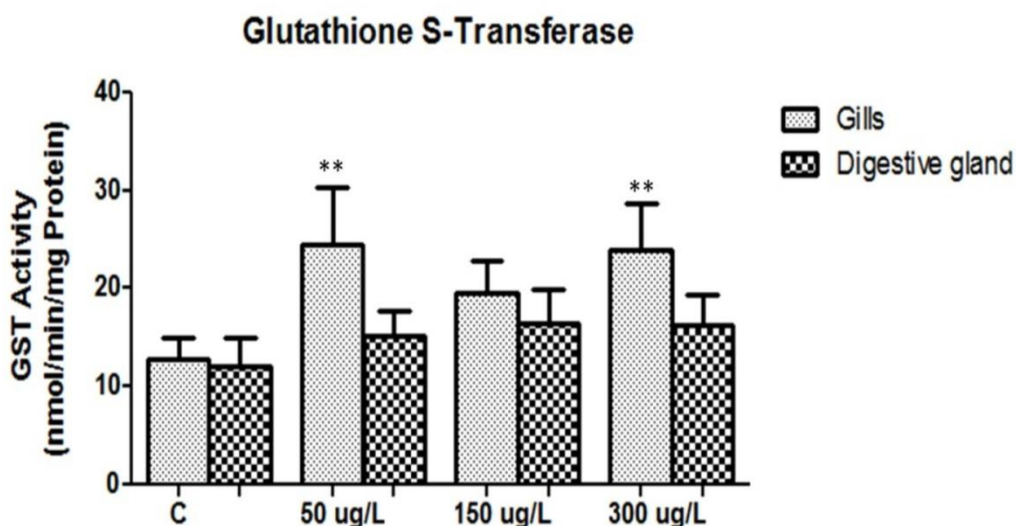


Figure 1. Glutathione transferase activity in gill and digestive gland of *M. edulis* exposed to copper. C: Control group. Superscripts of different letters are significantly different from gill control group (C) and group 50 µg/L at ** $p < 0.01$, and group 300 µg/L at ** $p < 0.01$.

Antioxidant enzyme activities such as those of CAT and GR have long been of interest as potential biomarkers in aquatic toxicology [35]. GR plays a key role in antioxidant defence by maintaining GSH levels in the cell and, in a field study with *M. galloprovincialis*, was found to increase with environmental exposure to various heavy metals [36]. Similarly, in the freshwater mussel, *Anodonta anatine*, GR activity also increased in response to copper exposure [37]. In the present study, GR activities decreased in gill after exposure to 150 µg/L copper chloride (Fig. 2) and no significant differences were observed for GR activity in digestive gland. This is consistent with mussels in the present study possessing adequate GSH levels to deal with the effects of the dose of copper used. In this regard, it is interesting that CAT activity was unaffected in both gill and digestive gland (Fig. 3). We previously found that *M. edulis* CAT activity did not vary with season in two separate sampling sites unlike other detoxification enzymes [38]. In a later study with mussels from the Baltic Sea, protein carbonylation, scope for growth and disulphide bridge patterns varied between sampling sites and copper exposure whilst CAT activity, again, did not vary [39]. This further supports the idea that animals experienced only modest oxidative stress at the copper doses used in this study.

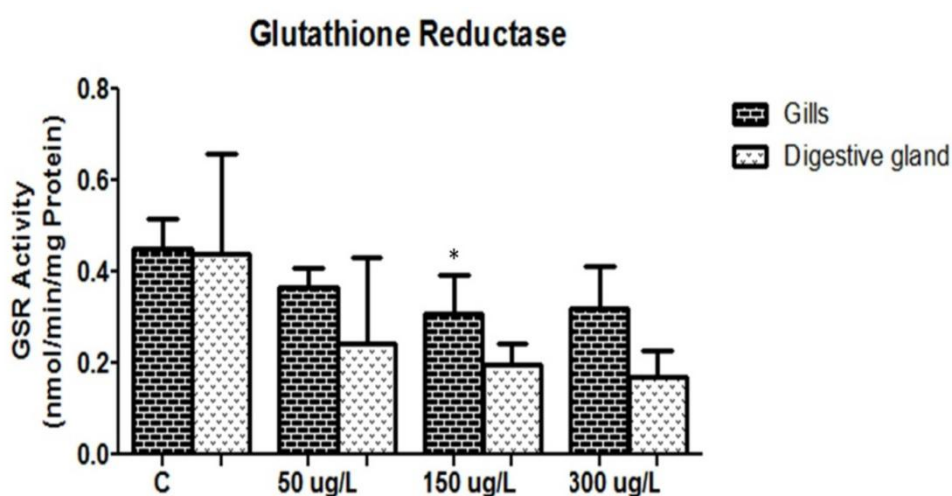


Figure 2. Glutathione reductase activity in gill and digestive gland of *M. edulis* exposed to copper. C: Control group. Superscripts of different letters are significantly different from gill control group (C) and group 50 µg/L at * p<0.05.

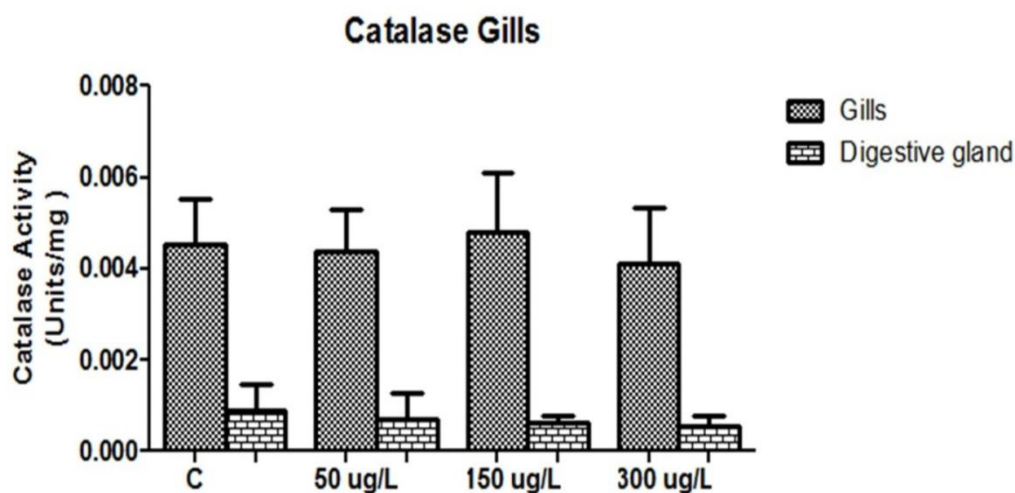


Figure 3. Catalase activity in gill and digestive gland of *M. edulis* exposed to copper. C: Control group.

Total protein thiols analysed by 1DE

Protein thiols obtained from extracts of gill (Fig. 4) and digestive gland (Fig. 5) were labelled with IAF and the proteins separated by 1DE. Gels were scanned for IAF fluorescence followed by staining with colloidal coomassie. This facilitates normalising total thiol fluorescence to total protein staining intensity for each electrophoresis lane yielding a relative measure of total protein thiol for each lane [22, 40]. Total protein thiols make an antioxidant contribution to the cell which is quantitatively similar to that of GSH [41]. Indeed, total protein thiols have previously been proposed as a novel biomarker of oxidative stress following PAH

treatment in mussels [42]. Protein thiol levels in digestive gland were found to be consistently slightly higher than those of gill (Fig. 6). This level was significantly elevated in gill at a dose of 150 $\mu\text{g/L}$ but then decreased markedly at the highest copper dose used, 300 $\mu\text{g/L}$. In contrast, total protein thiols of digestive gland only increased significantly at 300 $\mu\text{g/L}$. This suggests that gill and digestive gland respond differentially to the copper dose-range used here. Interestingly, it has previously been noted that the pattern of activity associated with variants of the antioxidant enzyme, Cu/Zn superoxide dismutase, in *M. edulis* varies between gill and digestive gland during the tidal cycle suggesting differing redox requirements for each tissue [43]. Proteomic analysis of the response to PAH exposure, revealed other stress-response proteins in addition to Cu/Zn superoxide dismutase, varying in *M. edulis* gill on immersion/emersion [44]. Our data suggest that protein thiol levels vary in a tissue-specific manner due to modest oxidative stress in response to the copper doses used in this study. This reflects the overall resilience of the proteome to copper-induced stress and led us to use 2DE to profile changes at the level of individual proteins.

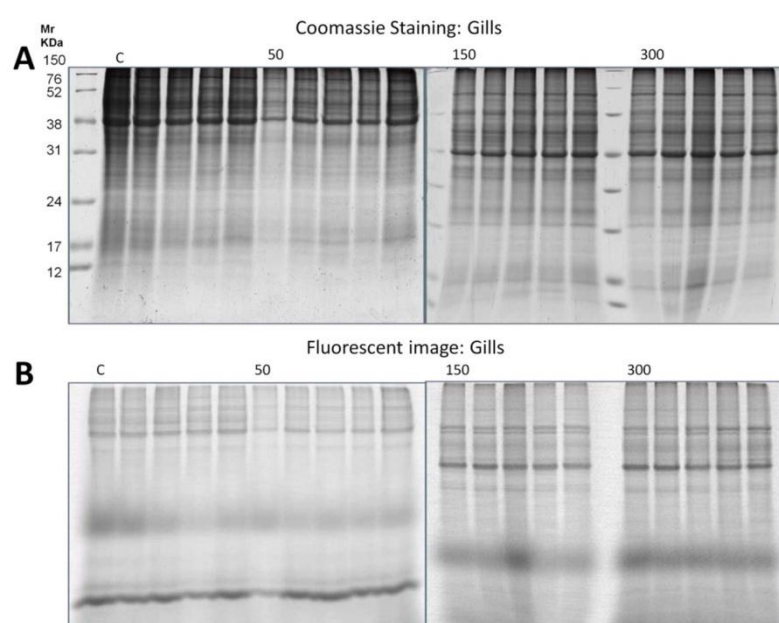


Figure. 4. 1DE protein profiles of gill cytosolic extracts from *M. edulis* exposed to copper. **(A)** Total protein visualised by colloidal coomassie staining. **(B)** Thiol-containing proteins visualised by IAF fluorescence. Lanes shown are for control (C), 50 $\mu\text{g/L}$, 150 $\mu\text{g/L}$ and 300 $\mu\text{g/L}$ copper. Molecular weight markers are shown (Mr) and 30 μg of protein from each lane was loaded for 5 biological replicates in each group.

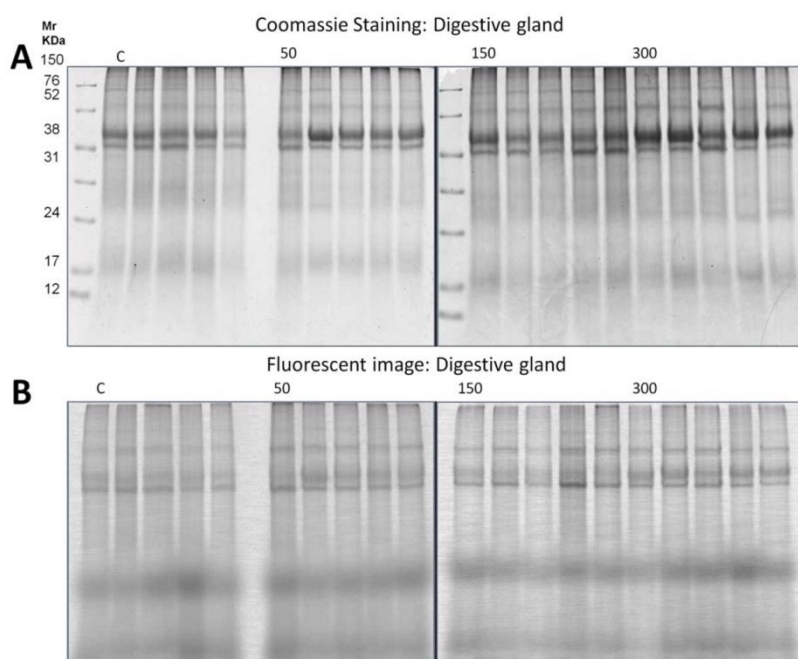


Figure. 5. 1DE protein profiles of digestive gland cytosolic extracts from *M. edulis* exposed to copper. **(A)** Total protein visualised by colloidal coomassie staining. **(B)** Thiol-containing proteins visualised by IAF fluorescence. Lanes shown are for control (C), 50 $\mu\text{g/L}$, 150 $\mu\text{g/L}$ and 300 $\mu\text{g/L}$ copper. Molecular weight markers are shown (Mr) and 30 μg of protein from each lane was loaded for 5 biological replicates in each group.

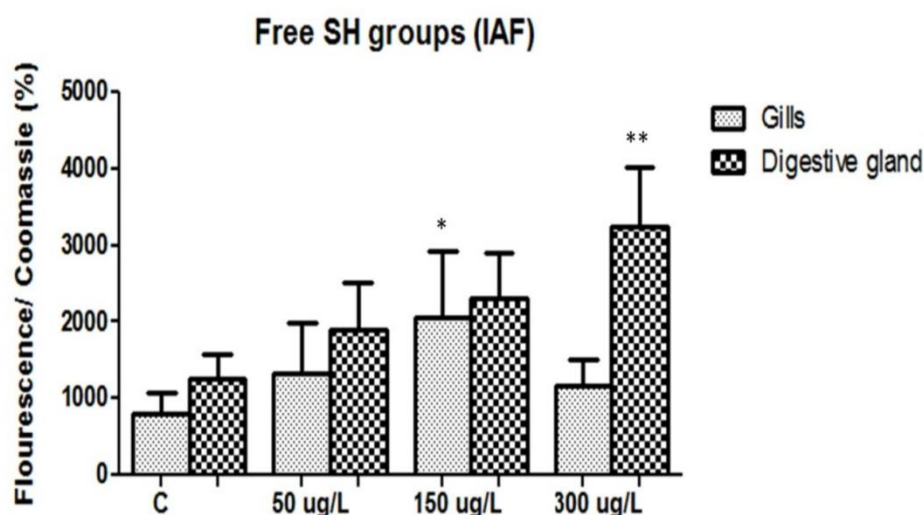


Figure 6. 1DE analysis of protein thiols (IAF) content in gill of *M. edulis* exposed to copper. Data are presented as mean \pm SD (n=5). Superscripts of different letters are significantly different from gills, control group and group 150 $\mu\text{g/L}$ * $p < 0.05$; and digestive gland, control group and group 300 $\mu\text{g/L}$ ** $p < 0.01$.

Proteomic analyses

Total gill proteins from the four treatment groups (control, 50 $\mu\text{g/L}$, 150 $\mu\text{g/L}$ and 300 $\mu\text{g/L}$) were separated by IEF in the pI range 3-10 and both IAF fluorescence and coomassie-stained images were obtained. These showed approximately 200 well-separated protein spots (Fig. 7) and analysis with SameSpots software revealed that 69 spots increased significantly in abundance in response to copper treatment whilst 13 decreased in abundance. Similarly, approximately 130 spots were evident in separations of IAF-labelled proteins (Fig. 8) of which 35 apparently increased in fluorescence intensity whilst 10 decreased in response to copper. Matching these gels by SameSpots software to a gel dataset previously obtained by us in a study of diclofenac effects on *M. edulis* gill [32] allowed identification of four coomassie-

stained and one IAF spots (Table 1; Table 2). Figure 9 shows illustrated zoom boxes of these. Only spot 407 (beta-tubulin) showed a decrease in intensity in this analysis with all other spots showing an increase in response to copper. A comparison of differences in response patterns for this panel of protein spots in response to copper and diclofenac [32] is instructive as it suggests that these pro-oxidants affect this small group of proteins differently. Spots 323 (calreticulin) and 99 (IAF-labelled heat shock cognate protein 70) both increased in response to copper but decreased in response to diclofenac whilst spots 407 (beta-tubulin), 546 (Arginine kinase) and 1128 (superoxide dismutase) increased in response to both pro-oxidants. This supports the idea that proteomics robustly detects protein changes in response to oxidative stress but may also be capable of profiling subtle differences between effects of different environmental pollutants. Because of the matching strategy adopted here, the relatively limited pattern of proteins shown in Fig. 9 should be regarded as a minimum PES since it excludes protein spots for which identifications were not available in this study.

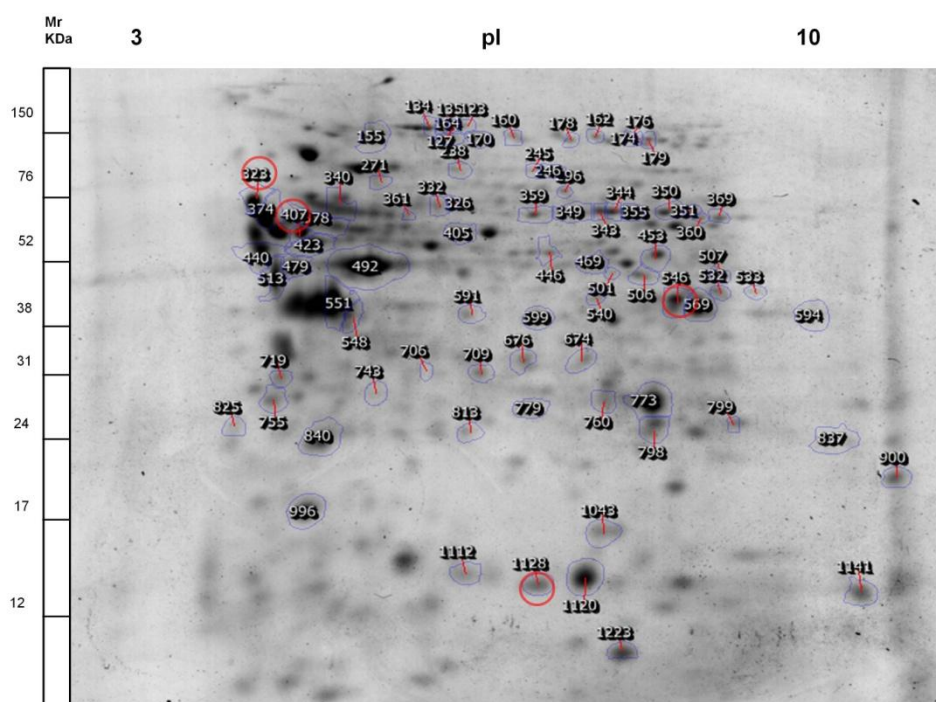


Figure 7. 2DE protein profiles of *M. edulis* gill cytosolic protein extracts. Total proteins were visualized by colloidal coomassie staining.

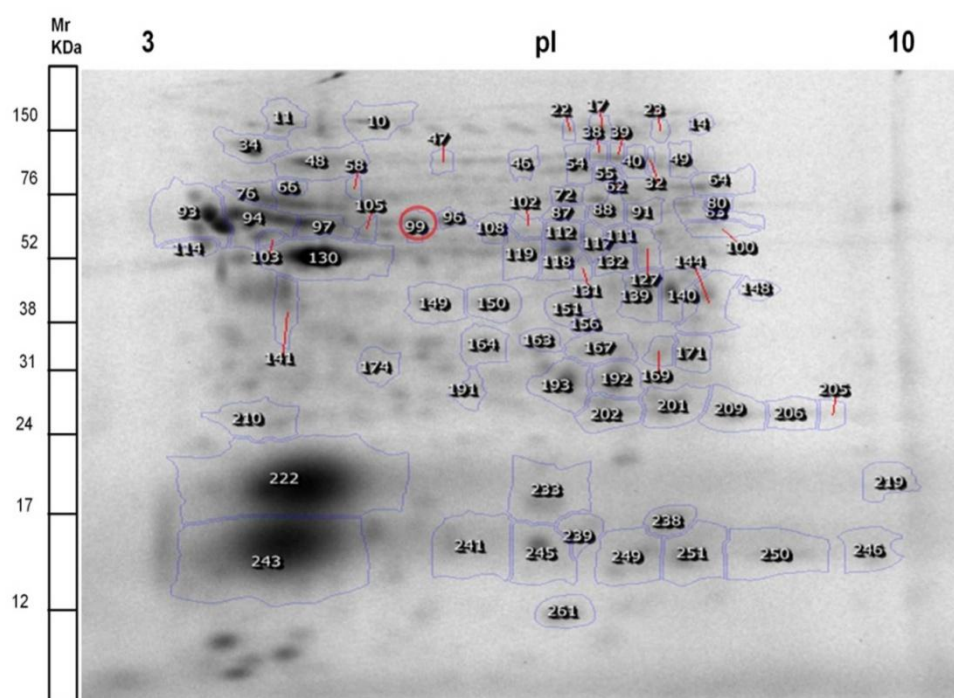


Figure 8. 2DE protein profiles of IAF-labelled *M. edulis* gill cytosolic protein extracts. Thiol-containing proteins were visualised by IAF fluorescence.

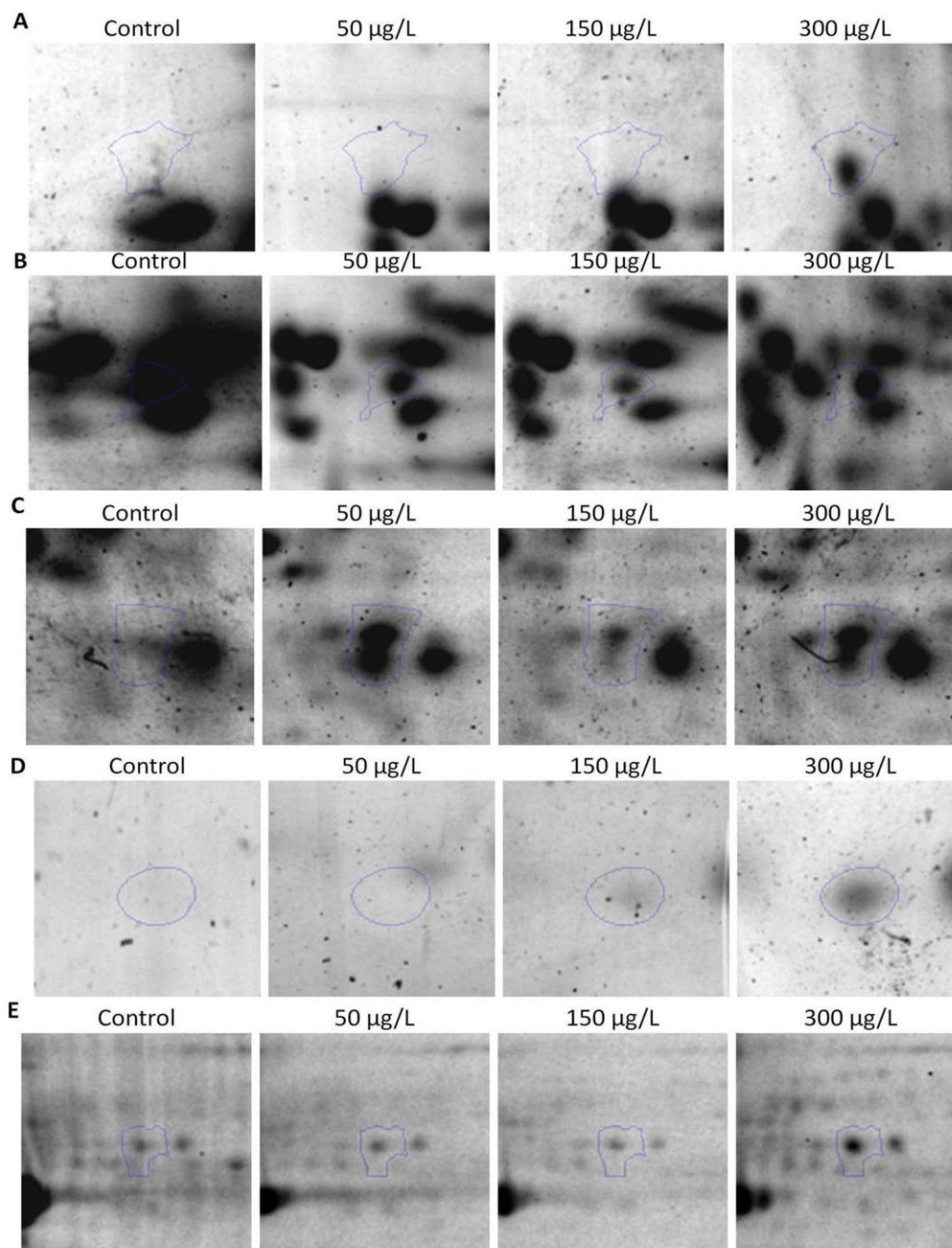


Figure 9. Representative 2DE images of gill extracts from *M. edulis* exposed to copper. Spots were identified by MALDI-TOF/TOF MS. Coomassie stained spots; (A) id: 323 (B) id: 407 (C) id: 546 (D) id: 1128 and (E). IAF-labelled spot (id: 99).

Table 1. Identified protein spots

Spot #	Accession #	Protein ID	Organism	Score	Expression
99	gi 77023195/ 17.55	Cytosolic heat shock cognate protein 70 (binding protein)	<i>Mytilus galloprovincialis</i> (Mediterranean mussels)	404/100	(IAF) Up-regulated
323	gi 156403953 / 42.62	Hypothetical protein; Calreticulin (associate with ER)	<i>Pinctada fucata</i> (Akoya pearl oyster)	96/99.032	Up-regulated
407	gi 34345525 5 gb A EM36067.1/ 18.10	Beta-tubulin (binding protein)	<i>Mytilus edulis</i> (Blue mussels)	131/100	Down-regulated
546	gi 228014684 / 18.74	Arginine kinase (energy metabolism)	<i>Aralius sp</i> DDM 2009 (subfamily <i>Oxycoryninae</i>)	197/100	Up-regulated
1128	gi 21526323 2 emb CAQ6 8509.1/ 15.77	Superoxide dismutase	<i>Mytilus galloprovincialis</i> (Mediterranean mussels)	447/100	Up-regulated

Table 2. Similarities of protein identification across chemical exposures.					
Protein	Acession number/ Mw (kDa)	Diclofenac exposure [31]	Expression	CuCl exposure	Expression
	Spot ID	Spot ID		Spot ID	
Cytosolic heat shock cognate protein 70 [<i>Mytilus galloprovincialis</i>]	gi 77023195/ 17.55	196	Down-regulated	99	Up-regulated
Hypothetical protein; Calreticulin [<i>Pinctada fukata</i>]	gi 156403953/ 42.62	299	Down-regulated	323	Up-regulated
Beta-tubulin [<i>Mytilus edulis</i>]	gi 343455255 gb AEM36067.1/ 18.10	362	Down-regulated	407	Down-regulated
Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	542	Up-regulated	546	Up-regulated
Superoxide dismutase [<i>Mytilus galloprovincialis</i>]	gi 215263232 emb CAQ68509.1/ 15.77	1072	Up-regulated	1128	Up-regulated
^a The protein score probability limit (where p< 0.05) is 85. ^b Peptides with confidence interval above 95% were considered. ↑ Fold change increased ↓ Fold change decreased in related to control mussels					

Proteins varying in response to copper exposure

Five *M. edulis* gill proteins were successfully identified in this study as being responsive to copper exposure (Fig. 9; Table 1). Of these, three were identified from genus *Mytilus* whilst the other two were identified, respectively, from an insect (*Aurelius sp.*) and an oyster (*Pinctada fucada*). This reflects the continuing limited representation of *Mytilus* genes in public domain databases [18].

IAF-labelled cytosolic heat shock cognate protein 70 (hsc 70) was previously identified as being oxidised on exposure to diclofenac [32]. This is a member of the heat shock protein 70 (hsp 70) family, one of the most conserved known protein families, which plays a key housekeeping role and is thought to be essential for life [44]. Unlike the stress-inducible hsp 70s, hsc 70 is constitutively expressed. In the present study, there was a significant increase in thiol fluorescence associated with this protein suggesting that copper-induced oxidative stress did not target thiol groups in this protein in contrast to diclofenac treatment where thiol oxidation of hsc 70 was observed [32]. Hsps have been extensively studied as stress biomarkers in environmental toxicology [46]. They are known to be responsive to a range of stressors in addition to heat and including oxidative stress [47]. During stress, hsps enter the nucleus where they protect proteins against denaturation [48]. Intriguingly, it has been reported that cysteine oxidation inactivates hsp70 whilst not affecting the closely-homologous hsc 70, showing that these proteins are differentially-sensitive to cellular redox status [49].

We previously reported both increased expression and enhanced oxidation of arginine kinase which suggested that this enzyme was highly redox-sensitive in response to diclofenac [32]. Arginine kinase is part of the phosphagen kinase protein

family which catalyses irreversible transfer of a phosphate group from arginine phosphate to ADP forming ATP [50]. In invertebrates, arginine phosphate and arginine kinase play roles analogous to creatine phosphate and creatine kinase in vertebrates and maintain a steady supply of ATP which is essential for the innate immune system and general metabolism [51]. The present observation suggests that this protein forms part of *M. edulis*'s general response to oxidative stress. Calreticulin was also found to be present in increased amounts here as was also previously reported for diclofenac exposure [32]. This protein is important in calcium homeostasis and also functions as a chaperonin in protein folding and forms part of the ER stress response [52]. Similarly, superoxide dismutase was significantly increased in abundance in response to both diclofenac and copper [32]. This is a key antioxidant enzyme which plays a major role in *M. edulis* being able to adapt to changes in oxygen concentration during the tidal cycle [43]. Taken together, the previously-mentioned three proteins most probably form part of a general response to oxidative stress as they behave similarly in their response to both diclofenac and copper. However, the structural protein beta tubulin which showed a decrease in abundance in response to diclofenac [32] in this study showed a significant increase in abundance. We previously discovered that this protein formed disulphide bonds in response to the pro-oxidant menadione [14]. In response to copper oxide nanoparticles ingested by *M. edulis*, beta tubulin was also found to alter in abundance [53]. These findings underline the redox-sensitive nature of beta tubulin in response to pro-oxidants.

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CHAPTER 5:

(Redox proteomic analysis of the effects of bisphenol A, a suspected endocrine disruptor, on *Mytilus edulis* gills)

**Redox proteomic analysis of the effects of bisphenol A, a suspected
endocrine disruptor on *Mytilus edulis* gills.**

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Keywords: BPA; biomarker; proteomics; mytilus; redox; ecotoxicology

Abstract

Bisphenol A is one of major anthropogenic chemicals that is widely present in the environment. It is also known as an endocrine disruptor which mainly used in polycarbonate plastics manufacturing. This contaminant gives toxic effects to marine organisms particularly bivalve. In this study, the blue mussel, *Mytilus edulis* was selected as the target organism. Mussels was exposed under controlled conditions to two different sub lethal doses of bisphenol A (50 ug/L and 500 ug/L) for 7 days with a week of recovery time. Enzyme activity parameters of gills homogenates were performed including glutathione transferase (GST), glutathione reductase (GR) and catalase (CAT). Oxidation of protein thiols was studied using one-dimensional electrophoresis (1DE) and two-dimensional electrophoresis (2DE). It was noted that there were significant differences in gill and digestive glands for glutathione transferase activities. There was significant different in gill glutathione reductase and catalase activities. Whilst, no effect was noted for digestive gland on glutathione reductase and catalase activities. Protein spots were detected by colloidal Coomassie staining and protein profiles compared with Progenesis SameSpots software. Additionally, these findings demonstrate that proteomics is a robust tool to extend valuable insights into possible mechanisms of toxicity of bisphenol A contaminants in mussels. Suggested that the results emphasize gills is the effective organ as a valuable discovery impacts pollution in mussels.

Introduction

Endocrine-disrupting pollutants represent an important category of anthropogenic chemicals commonly found in the environment. These are defined as exogenous chemicals or mixtures of chemicals that impact on endocrine system structure or function causing adverse effects (Witorsch, 2002; Flint, 2012). More than 66 chemicals have recently been identified by the European Commission as potential endocrine disruptors posing risks to humans (EC, 2015). Whilst the European Food Safety Authority and the UN's Food and Agricultural Organisation/World Health Organisation have discounted toxicity at current recommended doses (EFSA, 2010; FAO/WHO, 2010), concerns persist for human health risks, especially to the young, adolescents and pregnant women and the bisphenol A is now banned from the manufacture of babies' drinking bottles in Denmark and Canada. However, while laboratory studies point to endocrine disruptors being toxic to many fish species in laboratory studies, this seems not to have affected reproduction of fish species in the field (Mills and Chichester, 2005).

Bisphenol A (BPA) is a suspected endocrine disruptor (EC, 2015). It is one of the highest-volume chemical products produced worldwide (Crain et al., 2007) and is extensively used in food and beverage packaging, flame retardants, adhesives, building materials, electronic components, and paper coatings (Staples et al., 1998; Flint et al., 2012). It has been reported to cause several toxicological effects including endocrine dysfunction, reproductive failure, developmental abnormalities and decreased immunity (Zhou et al., 2010). BPA is acutely toxic to aquatic organisms in the concentration range 1000–10,000µg/L (Alexander et al. 1988; Goodman et al. 2006). The marine and aquatic environment is crucial for the study of BPA because discharge into the marine or aquatic environment may occur not

only through direct discharge of BPA-based products into estuaries, rivers and marine waters but also as effluent from wastewater treatment plants and landfill sites. Endocrine disruption effects have been reported for freshwater mussels (*Dreissena polymorpha*) exposed to municipal effluent (Quinn et al., 2004). Marine and aquatic organisms, especially sessile organisms like bivalves, may find it difficult to escape from BPA's toxic and endocrine-disruptive effects (Kang et al., 2007). BPA is rapidly absorbed and bioaccumulated by molluscs (Hayashi et al., 2008; Zhou et al., 2010), one of the largest and most widespread of animal phyla. Moreover, molluscs are used worldwide as sentinels of pollution in coastal marine environments (Fabbri et al., 2014). The use of biomarkers in such sentinels is a promising approach to risk assessment of potentially toxic chemicals (Xiangli et al., 2008). Redox proteomics which takes account not just of protein abundance changes but also of protein redox status changes, is a promising route to biomarker discovery (Chora et al., 2010; McDonagh et al., 2005; 2006). Relatively few redox proteomic studies have been performed on effects of chemical pollution in bivalves. In this study, we investigate the effects of BPA exposure on detoxification and antioxidant enzyme activities, protein thiol redox status, protein expression signatures (PES) and altered two-dimensional electrophoresis (2DE) patterns of proteins from *M. edulis* gill tissue. These studies offer novel insights into the biochemical consequences of BPA exposure.

Materials and methods

Chemicals

BPA, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), EDTA, phenyl-methylsulphonylfluoride (PMSF), reduced glutathione (GSH), oxidised glutathione (GSSG), 5-(iodoacetamido) fluorescein (IAF) and bisphenol A were purchased from Sigma Aldrich. All reagents used in 2DE were of analytical grade and obtained from Bio-Rad Ireland and GE Healthcare. All other reagents used were of analytical grade.

Animals and sample preparation

M. edulis (5-6 cm in length), were collected in early April 2013 from a pristine site (9° 30'W, 51°40'N) in Bantry Bay, County Cork, Ireland. This sampling location is well-known for commercial mussel aquaculture and water in this area is considered free of pollutants by European standards (Roycroft et al 2004). Approximately one hundred individual animals were collected from the intertidal zone at low tide and transported to the laboratory. The mussels were acclimated for a week in artificial sea water (ASW) at $10^{\circ} \pm 1^{\circ}$ C and the salinity was 32ppt with a 12-h light/dark cycle. Animals were fed daily with a commercial solution (Shellfish diet 1800) at the recommended 2% maximum feed conversion rate during acclimation and exposure. Bisphenol A was dissolved in dimethyl sulfoxide (DMSO) and added to the tanks after water change. Four groups were established for the experiment: control mussels (ASW only); solvent control (ASW+ 0.001% DMSO); 50µg/L (ASW+DMSO+ BPA); 500 µg/L (ASW+DMSO+ BPA). Each of the groups contained 5 biological

replicates with each replicate consisting of 5 pooled animals. The mussels were fed for 4 hours prior to exposure. Water was changed and the mussels were then exposed to BPA daily for 7 days. Mussels were then allowed to recover for a week before tissue dissection. No mortality was observed under the conditions used and all animals were observed to be feeding normally. Gill and digestive gland were dissected on ice and pooled in groups from five individual animals. The samples were homogenized in a motor-driven Teflon Potter-Elvehjem homogenizer in Tris-HCl buffer containing 10 mM Tris-HCl, 0.5 M sucrose, 0.15 M KCl, 1mM EDTA and 1mM PMSF, pH 7.2, at a weight: volume ratio of 1:3. The homogenate was centrifuged at 15 000 g for 1h at 4°C and the supernatant was collected and stored at -80°C for further analysis. The protein content was estimated by the Bradford method using BSA as a standard (Bradford, 1976).

Antioxidant activity

To investigate possible oxidative stress effects of BPA on the gills and digestive glands of *M. edulis*, antioxidant enzyme activities were measured. Glutathione transferase (GST) activity was quantified in gill and digestive gland tissues from sample aliquots containing 15 µg of protein diluted to a volume of 50 µl. Samples were loaded into a 96-well microtitre plate with 100 µl of 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) (from a 40 mM stock in ethanol) in 0.15 M potassium phosphate buffer (pH 6.5) (Habig et al, 1974; McDonagh and Sheehan, 2006). GST activity was measured spectrophotometrically by adding 50 µl of 20 mM reduced glutathione (GSH) and measuring absorbance at 340nm immediately and every 15 s for 5 min. The specific activity was expressed as nMol CDNB conjugate formed/min/mg protein; $\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Glutathione reductase (GR) activity was

determined using β -NADPH as substrate (Carlberg and Mannervik, 1985). The reaction rate was detected at 340nm and specific activity was expressed as nMol NADPH consumed/minute/mg protein; $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. Catalase activity (CAT) was measured according to the method of Aebi (1974). This is based on measuring decrease in absorbance at 240 nm due to the consumption of hydrogen peroxide (H_2O_2). Specific activity was expressed as U/min/mg protein; $\epsilon = 0.04 \text{ mM}^{-1} \text{ cm}^{-1}$.

Fluorescein (IAF) labelling

Protein thiols were labelled with 0.2 mM IAF from a 20 mM stock solution in dimethyl sulphoxide (Batey et al, 2002). Gill protein aliquots containing 150 μg protein (1DE) and 300 μg protein (2DE) were incubated with IAF for two hours on ice in the dark. Proteins were precipitated by incubating extracts in 10% trichloroacetic acid (TCA) for 5 min on ice, followed by centrifugation at 11,000 g for 3 min. The resulting pellet was washed in an excess of ice-cold acetone to remove TCA and any interfering salts or non-protein contaminants. Protein extracts were re-suspended in 75 μl sample buffer for 1DE (62.5 mM TrisHCl (pH 6.8) containing 25% glycerol, 2% SDS, 5% 2-mercaptoethanol and a trace amount of bromophenol blue) or 125 μl rehydration buffer for 2DE (7 M urea, 2 M thiourea, 2%) CHAPS, 4% ampholyte (Pharmalyte 3-10 pH), 1.2% DeStreak reagent and a trace amount of bromophenol blue). IAF reacts specifically only with reduced thiols (-SH) but not with oxidised variants such as sulphenic acids (-SOH) or disulphides (-S-S) which might be expected to form on oxidative stress (Baty et al., 2002).

One-dimensional electrophoresis (1DE)

1DE was performed in 12% polyacrylamide gels (Laemmli, 1970). Fluorescently-labelled proteins were resolved in polyacrylamide gels at a loading of 30 µg per lane, with four replicate lanes per sample. All proteins were dissolved in sample buffer (dH₂O; 0.5 M Tris-HCl, pH 6.8; glycerol; 10% SDS; 0.5% bromophenol blue), and electrophoresed (4°C) at 120V for two hours in running buffer (10X stock: 30.0g Tris base; 144.0g glycine; 10.0g SDS; 1L H₂O). Following electrophoresis, gels were scanned in a Typhoon scanner, model 9410 (Amersham Biosciences) with excitation wavelength set at 488 nm and emission wavelength at 520 ± 20 nm filter. Gels were then stained with colloidal coomassie followed by destaining (Dybala and Metzger, 2009). Gels were then scanned in a calibrated densitometer (BioRad, Hercules, CA, USA).

Two-dimensional electrophoresis

Homogenates (300 µg protein) were labelled with IAF as stated in the previous section. Protein pellets (150 µg protein) were resuspended in 125 µl rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 1.2% bis (2-hydroxyethyl)-disulfide, 4% ampholytes (3-10 for IEF, GE Healthcare) and a trace amount of bromophenol blue (Leung et al., 2011). The protein mixture was loaded onto an Immobiline DryStrip (pH 3-10 NL, 7cm, GE Healthcare), which was rehydrated overnight in the dark and this was followed by isoelectric focusing (IEF) using a PROTEAN IEF system (BioRad) according to the strip manufacturer's recommendations. After focusing, strips were reduced in equilibration buffer (6M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol) containing 2% dithiothreitol (DTT) for 20 min and thiols were blocked

with equilibration buffer containing 2.5% iodoacetamide for 20 min. The equilibrated strips were embedded in molten agarose (0.5%) containing trace bromophenol blue atop 12% SDS PAGE gels, and electrophoresed at 90V for 30 min. followed by a constant voltage (120 V) using a mini PAGE system (Atto, Tokyo, Japan) until the dye front reached the end of the gel cassette. Gels were washed three times with Millipore water before scanning and colloidal coomassie staining (Dyballa and Metzger, 2009). Four independent technical replicates of each 2DE gel were prepared. After electrophoresis, gels were scanned for fluorescence and then stained with colloidal coomassie as for 1DE. Image analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics Limited, UK) to identify significantly altered spots in each treatment group in response to the drug. This software employs an alignment-based analysis approach which maps the same number of spots across all gels in a single analysis. The data obtained from protein gel images were tested for normality prior to any analysis and significance testing level was set at 0.05. Principal component analysis and analysis of variance (one way ANOVA) were performed for comparison and assessment of statistically-significant expression and redox variation between treatments and control. The spots were considered significantly different when $p < 0.05$ according to ANOVA and a fold change of > 1.5 were evident. The 2DE images obtained in this study were matched with SameSpots software with 2DE separations obtained in our laboratory in a previous study of *M. edulis* exposure to diclofenac (Jaafar et al., 2015). This routine allowed rapid discovery of proteins previously-identified by mass spectrometry.

Data analysis

Background reduction and optical density quantification of protein bands in 1-D PAGE gels was performed using Quantity One image analysis software (Bio-Rad). The intensity of fluorescence values were normalised against protein content measured for the same electrophoresis lane. This measurement was performed to correct for slight differences in sample loading and enabled the extent of thiol oxidation to be compared between samples. Statistical analysis of enzymatic assay and 2DE were performed by one-way ANOVA. Homogeneity of variance was tested by Bartlett's method. Differences in variances and multiple comparisons were made by one-way analysis of variance followed by the Duncan *post hoc* test. All data are presented as means \pm SD.

Results

Antioxidant activity

GST activities were determined in both gill and digestive gland extracts of animals exposed to 50 and 500 $\mu\text{g/L}$ BPA as well as associated controls; control and solvent control (Fig. 1). There was a significant increase in GST activity in response to 50 and 500 $\mu\text{g/L}$ BPA in both tissues. GR was only increased in response to 500 $\mu\text{g/L}$ in gills from animals exposed to BPA but not at the lower dose and not in digestive gland (Fig. 2). Similarly, CAT activity was only elevated in gills from animals exposed to 500 $\mu\text{g/L}$ (Fig. 3). The statistical values for antioxidant enzyme activities are summarised in Supplementary Table 1.

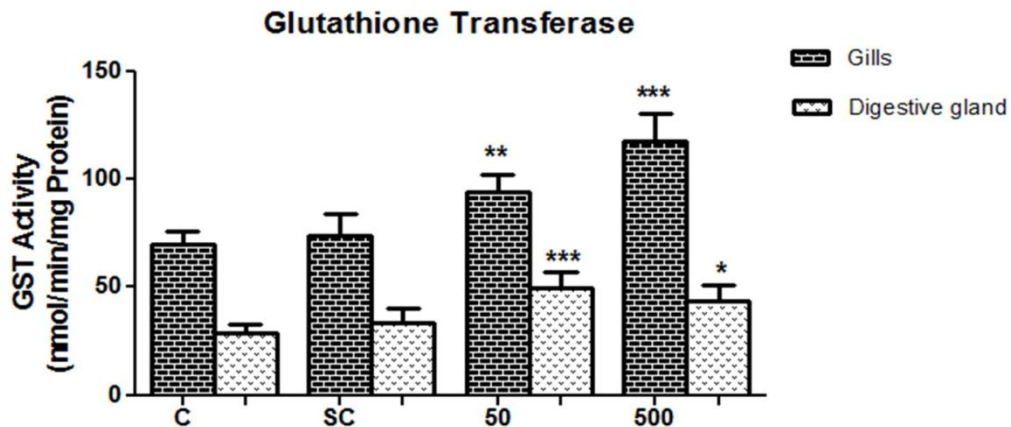


Figure 1. GST activity in tissues of *M. edulis* exposed to BPA. Data are expressed as mean \pm SD. C: Control group; SC: Solvent control. Superscripts of different letters denote significant difference from gill control group (C) and group 50 $\mu\text{g/L}$ at ** $p < 0.01$, and group 500 $\mu\text{g/L}$ at ** $p < 0.01$, digestive glands group 50 $\mu\text{g/L}$ at *** $p < 0.001$, and group 500 $\mu\text{g/L}$ at * $p < 0.05$.

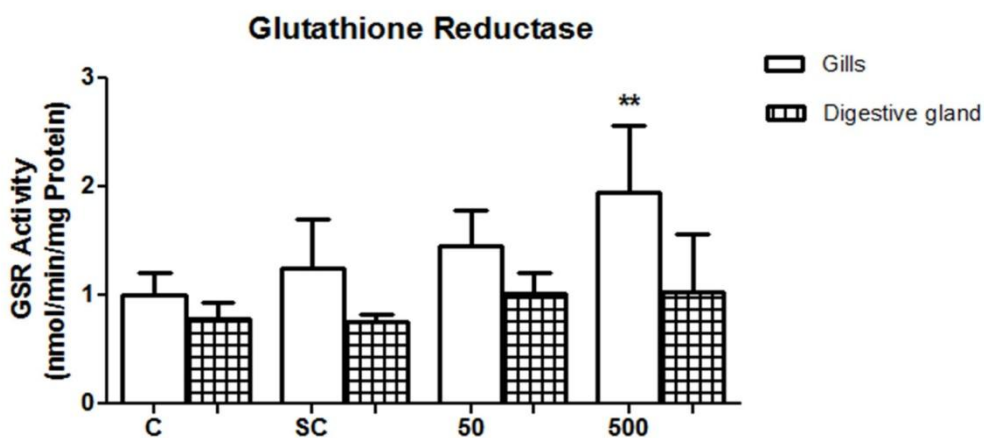


Figure 2. GR activity in tissues of *M. edulis* exposed to BPA. Data are expressed as mean \pm SD. C: Control group; SC: Solvent control. Superscripts of different letters are significantly different from gill control group (C) and group 500 $\mu\text{g/L}$ at ** $p < 0.01$.

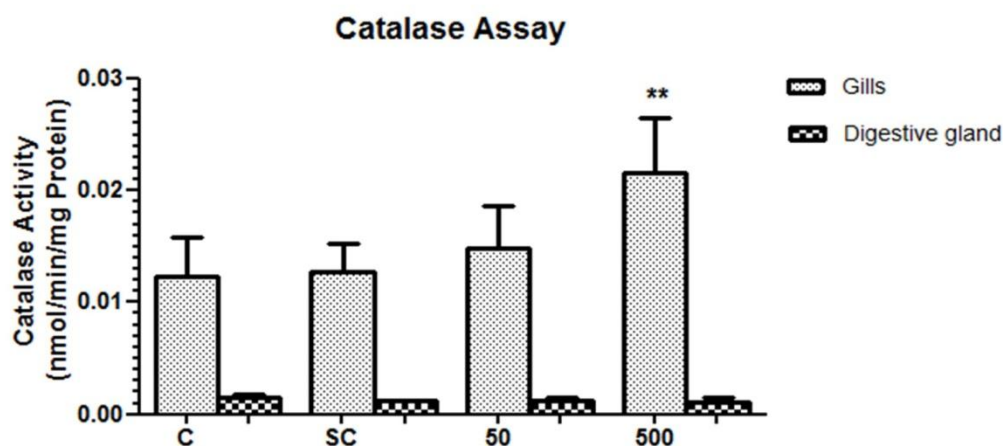


Figure 3. CAT activity in tissues of *M. edulis* exposed to BPA. Data are expressed as mean \pm SD. C: Control group; SC: Solvent control. Superscripts of different letters are significantly different from gill control group (C) and group 500 $\mu\text{g/L}$ at ** $p < 0.01$.

Proteomic analyses

Thiol groups of proteins were labelled with IAF and separated by 1DE. Gels containing proteins from gills (Figure. 4) and digestive gland (Figure. 5) were scanned for fluorescence and then stained with colloidal coomassie. This 1DE analysis revealed that protein thiol levels of digestive gland were higher than gill (Fig. 6) and that levels of gill and digestive gland protein thiols increased in the presence of 50 $\mu\text{g/L}$ and 500 $\mu\text{g/L}$ dose of BPA ($p < 0.001$).

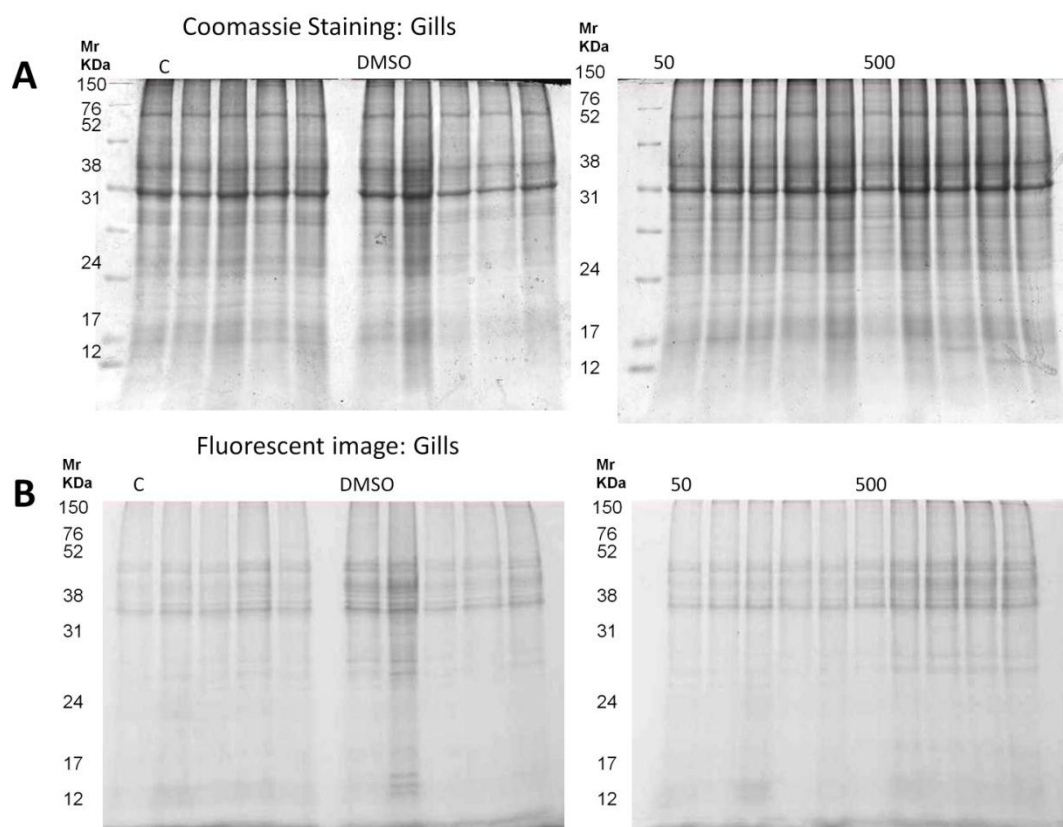


Figure. 4. 1DE of gill cytosolic protein extracts from *M. edulis* exposed to BPA. **(A)** Total protein levels by colloidal coomassie staining. **(B)** Fluorescence images of free thiol-containing proteins labelled with IAF. Gels contain molecular weight markers (Mr) and 30 µg of protein from each lane was loaded for 5 biological replicates of each Group: Control, DMSO, 50 µg/L and 500 µg/L.

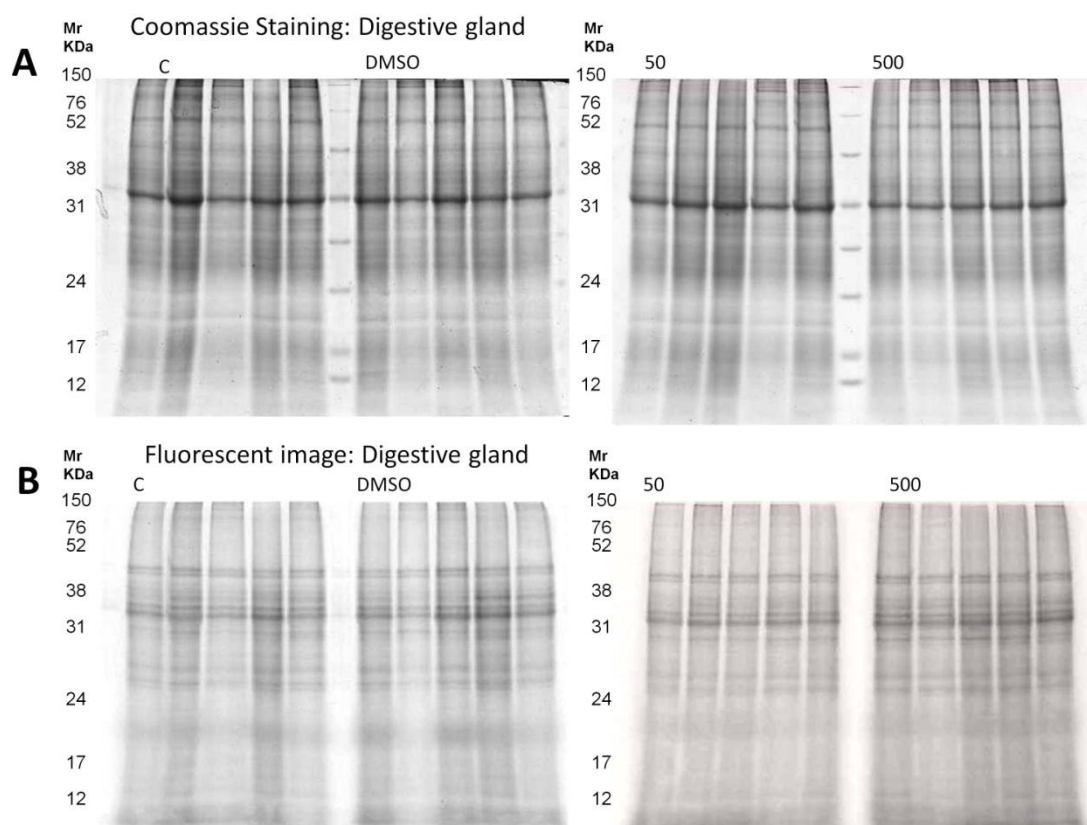


Figure. 5. 1DE of digestive gland cytosolic protein extracts from *M. edulis* exposed to BPA. **(A)** Total protein levels by colloidal coomassie staining. **(B)** Fluorescence images of free thiol-containing proteins labelled with IAF. Gels contain molecular weight markers (Mr) and 30 µg of protein from each lane was loaded for 5 biological replicates of each Group: Control, DMSO, 50 µg/L and 500 µg/L.

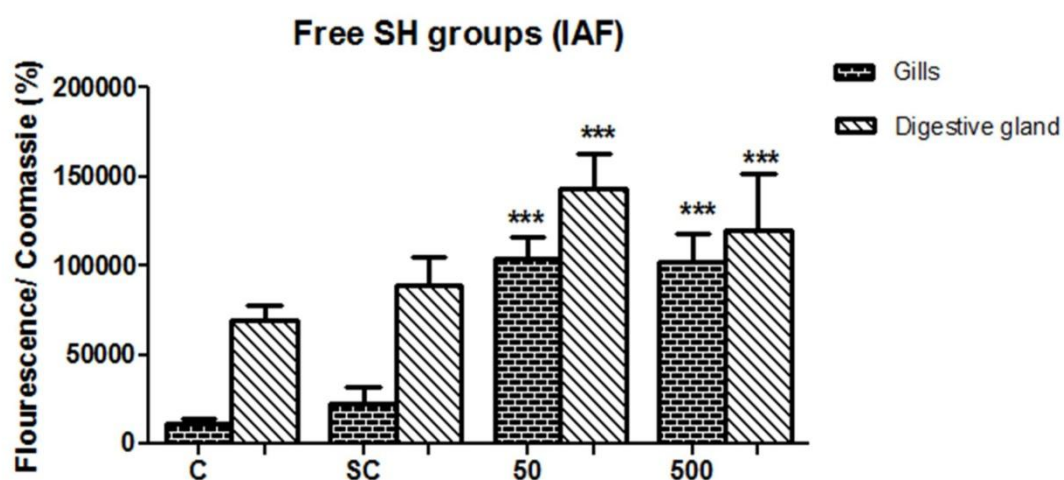


Figure 6. Ratio of IAF fluorescence to total protein from 1DE analyses. Data are presented as average \pm SD (n=5). Superscripts of different letters are significantly different from gills, control group and group 50 μ g/L and 500 μ g/L ***p<0.001; and digestive gland, control group and group 50 μ g/L and 500 μ g/L ***p<0.001.

The effects of differing dosages BPA were studied in more detail in *M. edulis* gill extracts by 2DE. Total proteins from the four groups (control, solvent control, 50 μ g/L and 500 μ g/L) were separated by IEF in the pI range 3-10 and both IAF fluorescence and coomassie-stained images were collected (Figs 7 and 8). [See also supplementary images 1 (Coomassie staining) and 2 (Fluorescent labelling) for complete 2DE images from all treatment groups]. These revealed well-separated protein spots. Analysis with SameSpots software allowed selection of alteration in specific spot abundance. The charts shown in Figs 9 and 10 give the number of altered and unaltered protein spots on BPA exposure. Of 58 total protein spots altering in response to BPA, 45 increased and 13 decreased. Of 19 altered IAF-labelled spots, 16 increased and 3 decreased.

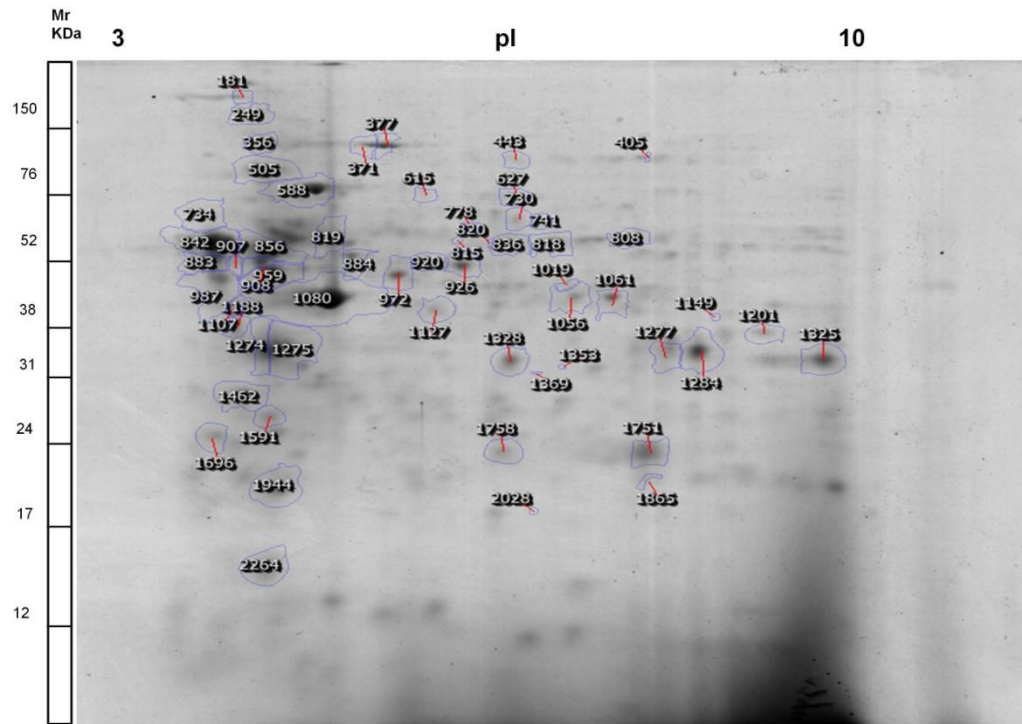


Figure 7. 2DE of gill cytosolic protein extracts from *M. edulis* (Control group) stained for protein. Total proteins were visualized by colloidal coomassie staining. 2DE was performed with 7cm non-linear pH 3-10 strips and in 12% SDS-polyacrylamide gels.

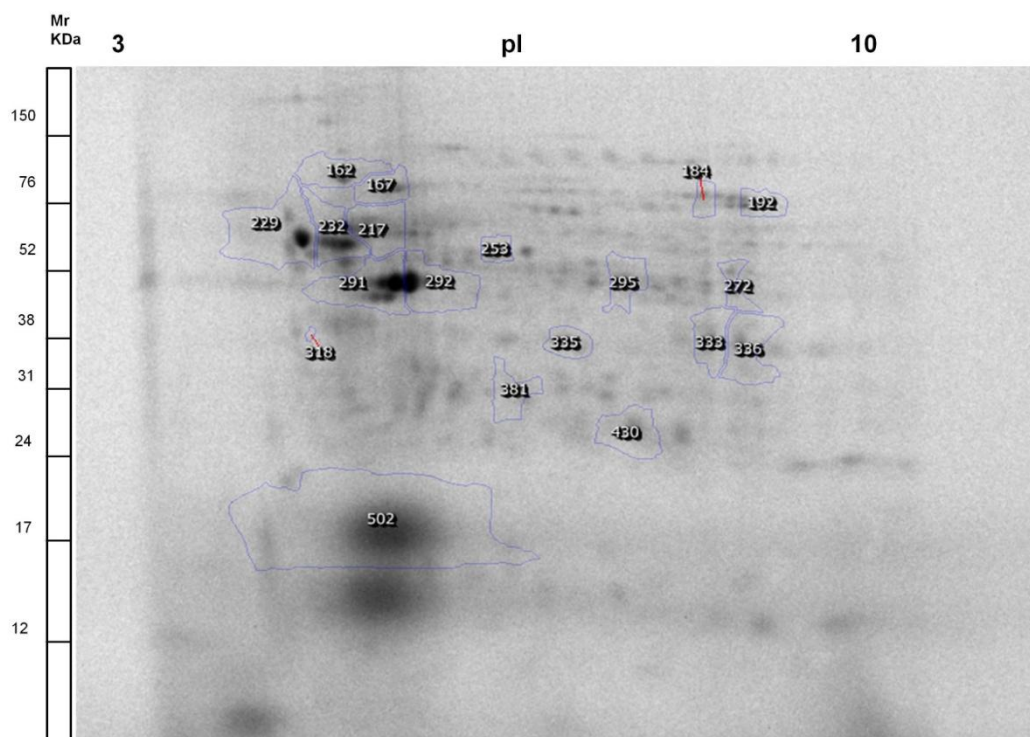


Figure 8. 2DE of IAF-labelled gill cytosolic protein extracts of *M. edulis* (Control group). 2DE was performed in 7cm non-linear pH 3-10 strips and 12% SDS-polyacrylamide gels.

Protein abundance (Coomassie Staining)

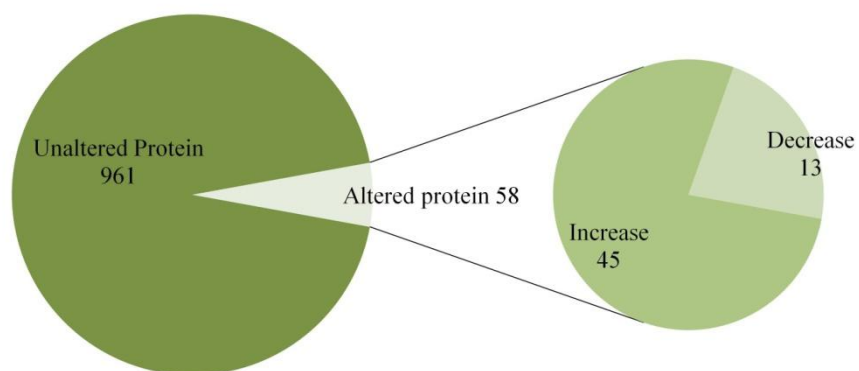


Figure 9. Summary of spot abundance changes on colloidal coomassie staining in 2DE of cytosolic protein extracts from gills of *M. edulis* exposed to BPA for 7 days.

Free thiols-containing protein (IAF Labelling)

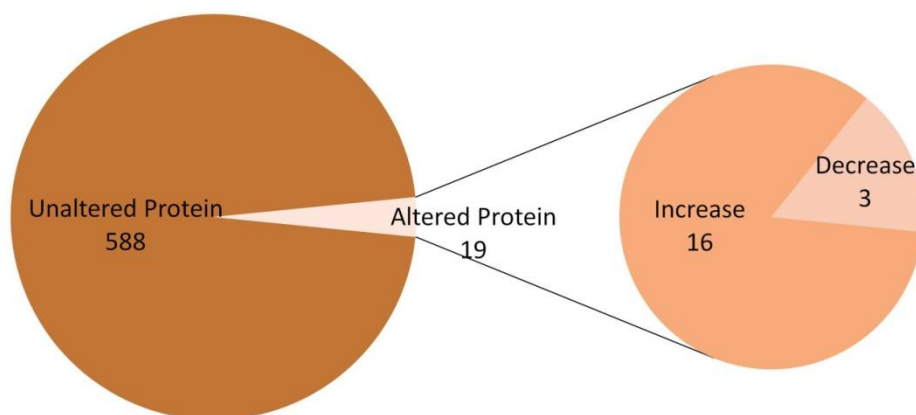


Figure 10. Summary of spot abundance changes on IAF fluorescence in 2DE of cytosolic protein extracts from gills of *M. edulis* exposed to BPA for 7 days.

Zoom windows for some selected spots are shown in Figs 11 and 12. Progenesis SameSpots software matching of gels in the present study with a gel dataset previously obtained for gills on diclofenac exposure (Jaafar et al., 2015) facilitated identification of proteins in six BPA-responsive spots. The proteins are; id 734 (Calreticulin), id 856 (Beta-tubulin), id 1277 (Arginine kinase), id 1275 (Heavy metal bind protein HIP), id 1274 (Heavy metal bind protein HIP) and id 1325 (Mg: bb02e05 protein coding). Details of the identifications are provided in Table 1.

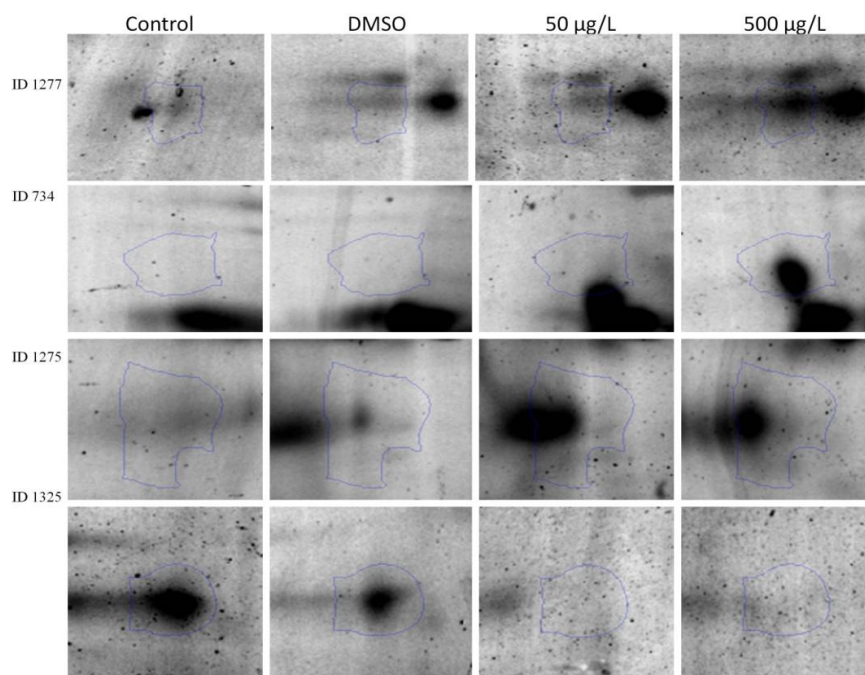


Figure 11. Representative 2DE zoom images of gill extracts from *M. edulis* exposed to BPA. Coomassie stained protein spots; id: 1277, id: 734, id: 1275 and id: 1325.

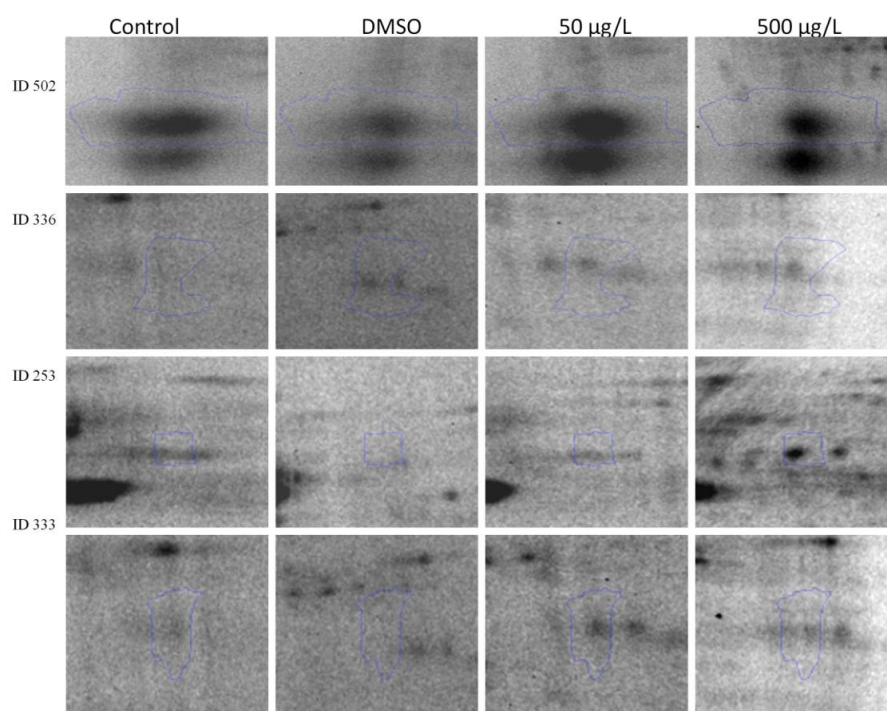


Figure 12. Representative 2DE zoom images of gill extracts from *M. edulis* exposed to BPA. IAF-labelled protein spots; id: 502, id: 336, id: 253 and id: 333.

Table 1. Similarities of protein identification across chemicals exposure.		
Diclofenac exposure (Jaafar et al., 2015)		This study: Bisphenol A
Spots ID	Protein	Spots ID
544	Heavy metal-binding protein HIP	1275
548	Heavy metal-binding protein HIP	1274
299	Calreticulin	734
362	Beta-tubulin	856
542	Arginine kinase	1277
566	Mg: bb02e05 (Protein coding)	1325

Discussion

BPA effects on antioxidant enzyme activities

Profiling antioxidant enzyme activities provides a useful approach to monitoring effects of environmental pollution as these activities function in a coordinated manner to protect organisms against direct and indirect toxicity (Kong et al., 2001; Valavanidis et al., 2006). GST activity has been extensively used as a biomarker for detecting stress in a wide range of organisms ranging from mammals to invertebrates (Han et al., 2013). In particular, they have been used as biomarkers in *M. edulis*, in assessing quality of the marine environment (Fitzpatrick et al., 1997). This is because, like many drug detoxification enzymes, GSTs are inducible in response to xenobiotics (Kong et al., 2001; Sheehan et al., 2001). In the present study, GST activity was found to increase in both gill and digestive gland in response to BPA. This is in agreement with observations in *M. galloprovincialis* (Arslan et al., 2014), the freshwater snail, *Bellamya purificata*, (Li et al., 2009) and the water-flea *Daphnia magna* (Jemec et al., 2012) where BPA exposure also significantly induced GST activity. In contrast, GR activity increased only in gills of animals exposed to 500 µg/L BPA. This is consistent with observation of poor responsiveness of this enzyme activity in *B. purificata* exposed to BPA (Li et al., 2009). Increases in both GST and GR activities suggest that these two enzymes could play roles in enhanced tolerance of oxidative effects in gill and help defend from BPA-induced damage. CAT activity also increased only in gill at the highest dose used. This is most probably in response to hydrogen peroxide due to direct binding of BPA compounds to –SH groups of the enzyme molecule (Crupkin and Menone, 2013). It can be concluded that GSTs were the most sensitive biomarker of the panel studied and that there is strong evidence that BPA causes significant xenobiotic stress to *M. edulis*,

especially in gill, the animal's main feeding organ. This is generally consistent with gill being the locus of a range of BPA-induced effects including micronuclei induction (Barsienne et al., 2006), tyrosine phosphorylation (Burlando et al., 2006) and DNA fragmentation (Taban et al., 2008).

BPA effects on total protein thiols

Cysteine is the second least-abundant residue in proteins but is often functionally conserved (Winterbourn 2008; Marino and Gladishev, 2010). Yet, quantitatively (perhaps because of protein abundance), protein thiols make a significant contribution to antioxidant defence by acting as a redox buffer (Hansen et al., 2009) and can be modified reversibly and irreversibly in response to oxidative insult (Lind et al., 2002). As BPA often causes oxidative stress, it might be expected that this compound could induce changes to protein thiol status. We have adapted the approach of Baty et al. (2002) involving tagging of protein –SH groups with IAF followed by electrophoretic separation and staining with colloidal coomassie. This allows normalisation of thiol signal to protein load in each gel lane and quantitation of total protein thiols in a large number of samples (Jaafar et al., 2015). In the present study it is evident that total protein thiols increased in both gill and digestive gland in response to BPA. This is consistent with the cells of both tissues responding to the oxidative insult posed by BPA by reducing thiols using defence systems such as glutaredoxins and thioredoxins (Fernandes and Holmgren, 2004). It is noteworthy that some flame retardants, a category of endocrine disruptors, such as tetrabromobisphenol A, are known to oxidise protein thiols while BPA has only moderate effects in this regard (Nakagawa et al., 2007). Taken together with the

observations of antioxidant enzyme effects above, the finding that protein thiols are increased in response to BPA supports that oxidative stress may induce a potent antioxidant response in *M. edulis*.

Proteomic analysis and responses

Proteomics is a robust and advanced toolset in ecotoxicology which has potential for identifying novel biomarkers and gaining insights into pollutant toxicity mechanisms (Dowling & Sheehan 2006; Vioque-Fernandez et al., 2009; Trapp et al., 2014). Environmental proteomic studies began when unique PES were described in 2DE separations of proteins from pollutant-exposed mussels (Shepard et al. 2000, Bradley et al. 2002). Previous biomarker investigations showed that *M. edulis* gills responded to oxidative stress after exposed to BPA. Further analysis using 2DE with Progenesis Samespots software analysis revealed that significant changes in abundance were observed. Six protein spots were noted to have similarities with proteins previously identified in a study of diclofenac exposure in our laboratory (Jaafar et al., 2015) which also mapped well to a 2DE reference map for *M. edulis* gill (Rocher et al., 2015). Five proteins were identified based on 1.3-3.2 fold increases and one protein decreased 1.3 fold in protein abundance in response to BPA exposure. Calreticulin (id: 734) acts as a chaperone in endoplasmic reticulum (ER) to support protein folding (Mendlovic et al., 2010). In this study, calreticulin showed a 3.2-fold increase which is consistent with a previous report that it is up-regulated and involved in protein refolding and the ER stress response (Leung et al., 2011). Beta-tubulin (id: 856) is a cytoskeletal protein often identified in marine organisms in response to contamination; clam (*Ruditapes decussates*), blue mussels (*Mytilus*

edulis), Medaka fish (*Oryzias latipes*) and abalone (*Haliotis diversicolor supertexta*) (Chora et al., 2009; Hu et al., 2014; Kim et al., 2007; Zhuo et al., 2010). In this study, beta-tubulin showed an increase in abundance. This protein is extensively involved in a range of cellular processes, reproduction and cell motility (Rocher et al., 2015). Arginine kinase (id 1277) belongs to the phosphagen kinase family which plays a key role in energy metabolism in invertebrates (Coyne, 2011). This enzyme may also yield information in environmental processing i.e. signalling molecules and interaction (Holt and Kinsey, 2002; Rocher et al., 2015).

Two spots were identified as Heavy metal-binding protein HIP (id 1274 and id 1275). These both increased in abundance on exposure to BPA. HIP protein has previously been found in gill tissue of blue mussels (Fields et al., 2012; Rocher et al., 2015) and is redox-sensitive (McDonagh and Sheehan, 2007). The major function of this protein in *M. edulis* gill is probably to bind divalent metal cations.

In conclusion, after 7 days exposure of *M. edulis* to BPA, gills showed extensive effects on antioxidant enzyme activity, especially GST, and an increase in total protein thiols. These responses increased with increasing BPA dosage. A number of proteins were identified by proteomic analysis as changing in response to BPA supporting the idea that the xenobiotic stress experienced in response to BPA had a component of oxidative stress.

Acknowledgements

Siti NurTahirah Jaafar received an Academic Training Scheme scholarship from Malaysian Government and Universiti Malaysia Terengganu. The authors thank Dr Maria O'Mahony, manager of the AFDC (Aquaculture and Fisheries Development Centre), School of Biological, Earth and Environmental Sciences, University College Cork, Ireland for facilitate the facility for exposure experimental work.

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Supplementary Table 1. Antioxidant activity measurements (mean \pm SD) from <i>M. edulis</i> gill and digestive gland. The groups are control (C), DMSO (SC), 50 μ g/L and 500 μ g/L Bisphenol A.				
Enzyme				
(U/mg)	C	DMSO	50 μg/L	500 μg/L
GST				
Gill	70.12 \pm 5.9600	74.022 \pm 10.1809	94.23 \pm 8.43205**	117.62 \pm 12.783***
Digestive gland	28.53 \pm 4.3900	33.673 \pm 6.3300	49.77 \pm 7.26578***	43.369 \pm 7.4437*
GR				
Gill	1.074 \pm 0.15163	1.378 \pm 0.4059	1.473 \pm 0.31812	2.245 \pm 0.3165**
Digestive gland	0.779 \pm 0.16019	0.752 \pm 0.07923	1.010 \pm 0.19916	1.025 \pm 0.54141
Catalase				
Gill	0.0123 \pm 0.00348	0.0127 \pm 0.002488	0.0148 \pm 0.00387	0.02157 \pm 0.00484**
Digestive gland	0.0014 \pm 0.000391	0.00122 \pm 0.00004336	0.00124 \pm 0.00027	0.001124 \pm 0.000329
* p < 0.05 ** < 0.01 *** p < 0.001				

Supplementary Table 2. List of spots detected by 2DE analysis of proteins base on protein abundance stained with Coomassie staining.

Spot number	Anova (p)	Fold	BPA exposure
1277	0.015	1.9	Increase
1056	0.026	1.4	Increase
778	0.033	1.5	Increase
734	0.053	3.2	Increase
1019	0.059	1.6	Increase
405	0.059	2.2	Increase
907	0.073	1.7	Increase
908	0.089	1.9	Increase
615	0.118	1.4	Increase
1188	0.123	1.8	Increase
1275	0.171	1.6	Increase
1061	0.184	1.5	Increase
972	0.203	1.5	Increase
819	0.206	1.5	Increase
181	0.295	5	Increase
249	0.296	1.6	Increase
818	0.301	1.4	Increase
926	0.303	1.5	Increase
884	0.345	1.5	Increase
1127	0.354	1.3	Increase
920	0.359	1.3	Increase
741	0.37	1.4	Increase
1201	0.374	1.4	Increase
1080	0.381	1.2	Increase
883	0.384	1.4	Increase
959	0.407	1.4	Increase
836	0.42	1.2	Increase
1751	0.444	1.6	Increase
808	0.477	1.4	Increase
588	0.515	1.6	Increase
1274	0.557	1.3	Increase
505	0.579	1.6	Increase
856	0.625	1.3	Increase
987	0.649	1.2	Increase
1284	0.708	1.2	Increase
1944	0.723	1.2	Increase
2264	0.768	1.2	Increase
356	0.795	1.4	Increase
730	0.848	1.1	Increase
377	0.875	1.3	Increase
371	0.878	1.5	Increase
627	0.88	1.1	Increase
1696	0.892	1.2	Increase
1758	0.944	1.2	Increase
1328	0.957	1	Increase
1353	0.032	1.9	Decrease
1369	0.147	1.9	Decrease

820	0.147	2.1	Decrease
1107	0.171	2.7	Decrease
2028	0.311	1.6	Decrease
1149	0.514	1.6	Decrease
815	0.594	1.4	Decrease
842	0.715	1.3	Decrease
1462	0.808	1.3	Decrease
1325	0.825	1.3	Decrease
1591	0.838	1.2	Decrease
443	0.855	1.2	Decrease
1865	0.873	1.3	Decrease

Supplementary Table 3. List of spots detected by 2DE analysis of proteins labelled with 5-iodoacetamidofluorescein (IAF).

Spot number	Anova (p)	Fold	BPA exposure
167	0.002	1.5	Increase
229	0.008	1.8	Increase
272	0.011	1.7	Increase
336	0.029	1.4	Increase
291	0.08	1.3	Increase
295	0.087	1.3	Increase
430	0.116	1.3	Increase
381	0.173	1.4	Increase
192	0.19	1.4	Increase
162	0.203	1.3	Increase
232	0.264	1.5	Increase
184	0.326	1.3	Increase
333	0.499	1.2	Increase
318	0.627	1.3	Increase
253	0.905	1.1	Increase
217	0.979	1.1	Increase
292	0.979	1.1	Decrease
502	0.028	1.4	Decrease
335	0.081	1.3	Decrease

CHAPTER 6: DISCUSSION

6. General discussion

The biological effects of pollutants in marine organisms can vary greatly between species. These differences arise from variation in the pattern of exposure, routes of uptake, metabolism following uptake, rates and sites of accumulation and sensitivity of the target organs (Rhind, 2009). The marine bivalve, *M. edulis* was selected for the present study. The principal objective of this thesis was to exploit valuable information provided by traditional biomarker measurements combined with a more novel redox proteomics approach to study of anthropogenic pollutant effects on *M. edulis*. As a sessile and widely-distributed filter-feeder, this genus has found huge acceptance in ecotoxicology. By April 2015, Web of Science had 5,760 records of publications using either “ecotoxicology” or “toxicology” and “Mytilus” as search terms. Anthropogenic pollution has high potential to induce ROS and many studies of traditional biomarkers have focused on aspects of ROS production and detoxification (Livingstone, 2001).

Excessive ROS production triggered by pollutants may lead to oxidative stress in aquatic organisms, damaging proteins and other important cell components (McDonagh and Sheehan, 2007; Lemos et al., 2010). In oxidative stress, more than 70% of ROS are absorbed by proteins, resulting in a wide variety of structural modifications in amino acid side-chains (Davies, 2005). Therefore, oxidative stress has the potential to introduce extensive complexity into proteomes as covalent modifications representing new structural variants of proteins. These post-translationally modified protein variants would be expected to behave differently in electrophoresis and mass spectrometry systems, making analysis and identification more complex (Butterfield et al., 2014; Kim et al., 2015). Moreover, it is also likely that oxidative modifications will alter the activity, stability and signalling potential

of individual proteins (Levonen et al., 2004; Winterbourn, 2008). Redox proteomics methods focus on using proteomics to elucidate redox-modulated changes to the proteome (Sheehan et al., 2010; Butterfield et al., 2014; Kim et al., 2015). These studies include changes in levels of specific proteins within the proteome or probing post- translational redox-based changes in protein structure. Mass spectrometry has obvious and fundamental relevance to these studies (Butterfield et al., 2014; Trapp et al., 2014; Kim et al., 2015).

6.1 Antioxidant enzymes

To make connections between proteomics results and standard biochemical measurement of oxidative stress, enzyme activities of GST, GR and CAT were measured. These enzymes are often associated with oxidative stress and yet also often feature in proteomics-derived protein lists as being responsive to pollutants (Apraiz et al., 2006; McDonagh and Sheehan, 2007; Maria et al., 2013). This analysis was combined with measurement of protein thiol content using the fluorescent dye IAF which allows us to follow –SH-containing proteins in electrophoretic separations (Baty et al., 2002). GSTs play key roles in cellular defence against xenobiotics and oxidative damage by catalyzing the conjugation of GSH through its cysteine thiol, with electron-deficient carbons in the second substrate (Sheehan et al., 2001; Han, 2013; Mashiyama et al., 2014). GSTs are predominantly cytosolic defence systems which can catalyse a wide range of chemical reactions additional to conjugation such as reductive dechlorination (Crupkin & Menone, 2013; Mashiyama et al., 2014). Interestingly, across three different and independent chemical exposures (diclofenac, copper and BPA), only diclofenac exposure did not show any significant difference in GST response.

Meanwhile, Copper and bisphenol A showed significant increases in gill. GR plays a key role in oxidative stress tolerance by maintaining high levels of GSH and a high GSH:GSSG ratio (Carlberg and Mannervik, 1985). GR directly reduces GSSG to GSH with associated oxidation of NADPH (Baker et al., 1996). The supply of NADPH is maintained by diversion of glucose to the pentose phosphate pathway (Barcia-Vieitez and Ramos-Martinez, 2014). In fact, under oxidative stress conditions, cells divert a significant part of their energy budget into production of NADPH which is a substrate also for other enzymes such as thioredoxin reductase, the enzyme that reduces the antioxidant protein thioredoxin (Sengupta and Holmgren, 2013). GR activity measurements suggest that all three of chemicals gave significant responses on gills. CAT is important in the prevention of oxidative stress damage and is responsible for detoxifying hydrogen peroxide (Jemec et al., 2010). This enzyme possesses an active site thiol which is capable of oxidation by pro-oxidants with consequent inactivation (Hu et al., 2010). If the activity of this enzyme decreases, this could indicate damage in response to oxidative stress (Halliwell & Gutteridge 2007; Jemec et al., 2010). In this study, CAT showed significant differences in digestive gland exposed to diclofenac and in gills exposed to BPA, but showed no significant difference for both tissues on copper exposure. Taken together, a pattern of tissue- and chemical-specific response is evident across the three enzymes studied with clear implications that oxidative stress is occurring, especially in gill.

6.2 Redox proteomics

To better explain the effects of anthropogenic chemical pollution (diclofenac, copper and bisphenol A) and to have better comparison between enzyme assay data and

specific changes in tissues, redox proteomics was applied. This enables easier comparison across proteins altering in different experiments. All gel images obtained with a single stain (colloidal coomassie) were aligned to a single representative reference gel image, which was chosen for practical and image quality reasons within the anthropogenic chemical. Gel images for IAF-labelled proteins (which had been captured prior to coomassie staining) were similarly aligned to investigate oxidative changes to thiol groups in proteins. Collectively, protein thiols are a key redox buffer system in cells and, in this regard, are more quantitatively important than GSH (Hansen, 2009). Measurements of total protein thiols were made by normalising total IAF fluorescence intensity to protein staining intensity for each track in 1DE separations (Hu et al., 2014; Jaafar et al., 2015). This facilitated analysis of multiple biological and analytical replicates. There was quite a bit of variation in this parameter with, for example, control digestive gland sometimes possessing higher levels than control gill (Jaafar et al., 2015) but lower levels in the experiments where copper and BPA were used. In the case of diclofenac, total protein thiols were significantly decreased at both doses used in gill but not in digestive gland which is consistent with oxidation of protein thiols (Jaafar et al., 2015). However, on exposure to BPA, total protein thiols increased in both gill and digestive gland whilst copper exposure caused an increase in gill at an intermediate dose and also an increase in digestive gland at the highest dose used. These observations are puzzling as a decrease would be expected in all cases of oxidative stress. It is possible that BPA triggers an increase in antioxidant status of gills perhaps by *nrf1* and *nrf2* factors through their *cis*-regulatory antioxidant response elements. It has been demonstrated recently in cultured HEK 293 cells that BPA exposure created a more reduced intracellular environment which would be expected

to reduce protein thiols (Chepelev et al., 2013). These effects of BPA are thought to be highly dose-dependent (Quesnot et al., 2014). Therefore, these data suggest that protein thiols may be an informative global observation within experiments but should be interpreted with caution across different exposures.

Table 1 shows the similarities in protein identifications across chemical exposures in this study and Table 2 shows fold-changes in specific spots. Venn diagram (Fig. 1) showed the summarise results of the protein identified from three different exposure. Including all of the chemical exposures, three proteins behaved in similar and consistent ways; calreticulin, beta-tubulin and arginine kinase. There were eight protein similarities between diclofenac and BPA exposure (calreticulin, beta-tubulin and arginine kinase, heavy metal binding protein HIP and Mg: bb02e05 [Protein coding]). Five proteins were showed similarity between diclofenac and copper exposure (cytosolic heat shock cognate protein 70, calreticulin, beta-tubulin, arginine kinase and superoxide dismutase). The most abundant protein identified across the three treatments used was arginine kinase. This is an important observation as this enzyme is key to maintaining ATP levels in invertebrates (Coyne, 2011) and this could mean that this crucial aspect of energy metabolism in *M. edulis* is redox-sensitive. This is a novel and previously-unknown observation. This enzyme has been purified from lobster and other invertebrates (Blethen and Kaplan, 1967), and crystal-derived and NMR structures are available from crab and other marine invertebrates in the protein databank (Niu et al., 2011). On diclofenac treatment, two IAF-labelled spots and one coomassie-stained protein spots were identified as arginine kinase (Jaafar et al., 2015). Cytosolic heat shock cognate protein 70 was also identified as an IAF- labelled spot.

The use of a redox proteomics approach enabled detection of protein changes across the various chemical exposures in this study. Reliance was placed on matching of spot patterns across the three datasets of gels using SameSpots software. Moreover, It was discovered that there was generally good agreement between spot patterns and distributions in this study (Jaafar et al., 2015) and a recently-published reference map for *M. edulis* gill (Rocher et al., 2015). This gave further confidence in identifications yielded by the spot-matching strategy adopted here. It seems that the redox proteomics approach taken here was sufficiently robust to identify redox targets of thiol modification in response to xenobiotic-induced stress (McDonagh & Sheehan, 2007).

Table 1. Similarities of protein identification across chemical exposures.				
Protein	Acession number/ Mw (kDa)	Diclofenac exposure (Jaafar et al., 2015)	Bisphenol A exposure	Copper chloride exposure
		Spot ID	Spot ID	Spot ID
Cytosolic heat shock cognate protein 70 [<i>Mytilus galloprovincialis</i>]	gi 77023195/ 17.55	196 fluorescent	-	99
Arginine kinase [<i>Cuphodes sp. 6 ex Diospyros kaki/Diospyros japonica/Diospyros lotus</i>]	gi 299819008/ 20.43	310 fluorescent	333 fluorescent	-
Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	311 fluorescent	336 fluorescent	-
Heavy metal-binding protein HIP	gi 46395578 sp P83425.1 HIP_MYTED/ 24.27	544	1275	-
Heavy metal-binding protein HIP	gi 46395578 sp P8 3425.1 HIP_MYTE D/ 24.27	548	1274	-
Calreticulin (<i>Pinctada fucata</i>)	gi 156403953/ 42.62, gi 150404776	299	734	323
Beta-tubulin [<i>Mytilus edulis</i>]	gi 343455255 gb A EM36067.1/ 18.10	362	856	407
Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	542	1277	546
Mg: bb02e05 (Protein coding) [<i>Xenopus laevis</i>]	gi 148223127/ 35.88	566	1325	-
Superoxide dismutase [<i>Mytilus galloprovincialis</i>]	gi 215263232 emb CAQ68509.1/ 15.77	1072	-	1128

Table 2. Fold changes between chemical exposures.				
Protein	Acession number/ Mw (kDa)	Diclofenac exposure (Jaafar et al., 2015)	Bisphenol A exposure	Copper chloride exposure
		Fold change	Fold change	Fold change
Cytosolic heat shock cognate protein 70 [<i>Mytilus galloprovincialis</i>]	gi 77023195/ 17.55	↓ ; -1.5 (IAF)	-	↑ ; 1.4 (IAF)
Arginine kinase [<i>Cuphodes sp. 6 ex Diospyros kaki/Diospyros japonica/Diospyros lotus</i>]	gi 299819008/ 20.43	↓ ; -1.3 (IAF)	↑ ; +1.2 (IAF)	-
Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	↓ ; -1.3 (IAF)	↑ ; +1.4 (IAF)	-
Heavy metal-binding protein HIP	gi 46395578 sp P83425.1 HIP_MYTED/ 24.27	↓ ; -1.7	↑ ; +1.6	-
Heavy metal-binding protein HIP	gi 46395578 sp P8 3425.1 HIP_MYTE D/ 24.27	↓ ; -1.7	↑ ; +1.3	-
Calreticulin (<i>Pinctada fucata</i>)	gi 156403953/ 42.62, gi 150404776	↓ ; -2.8	↑ ; +3.2	↑ ; +2.5
Beta-tubulin [<i>Mytilus edulis</i>]	gi 343455255 gb A EM36067.1/ 18.10	↓ ; -2.2	↑ ; +1.3	↓ ; -1.9
Arginine kinase [<i>Aralius sp. DDM-2009</i>]	gi 228014684/ 18.74	↑ ; +2.0	↑ ; +1.9	↑ ; +1.9
Mg: bb02e05 (Protein coding) [<i>Xenopus laevis</i>]	gi 148223127/ 35.88	↓ ; -1.7	↓ ; -1.3	-
Superoxide dismutase [<i>Mytilus galloprovincialis</i>]	gi 215263232 emb CAQ68509.1/ 15.77	↑ ; +2.0	-	↑ ; +2.6
↑ Fold change increased ↓ Fold change decreased in related to control mussels				

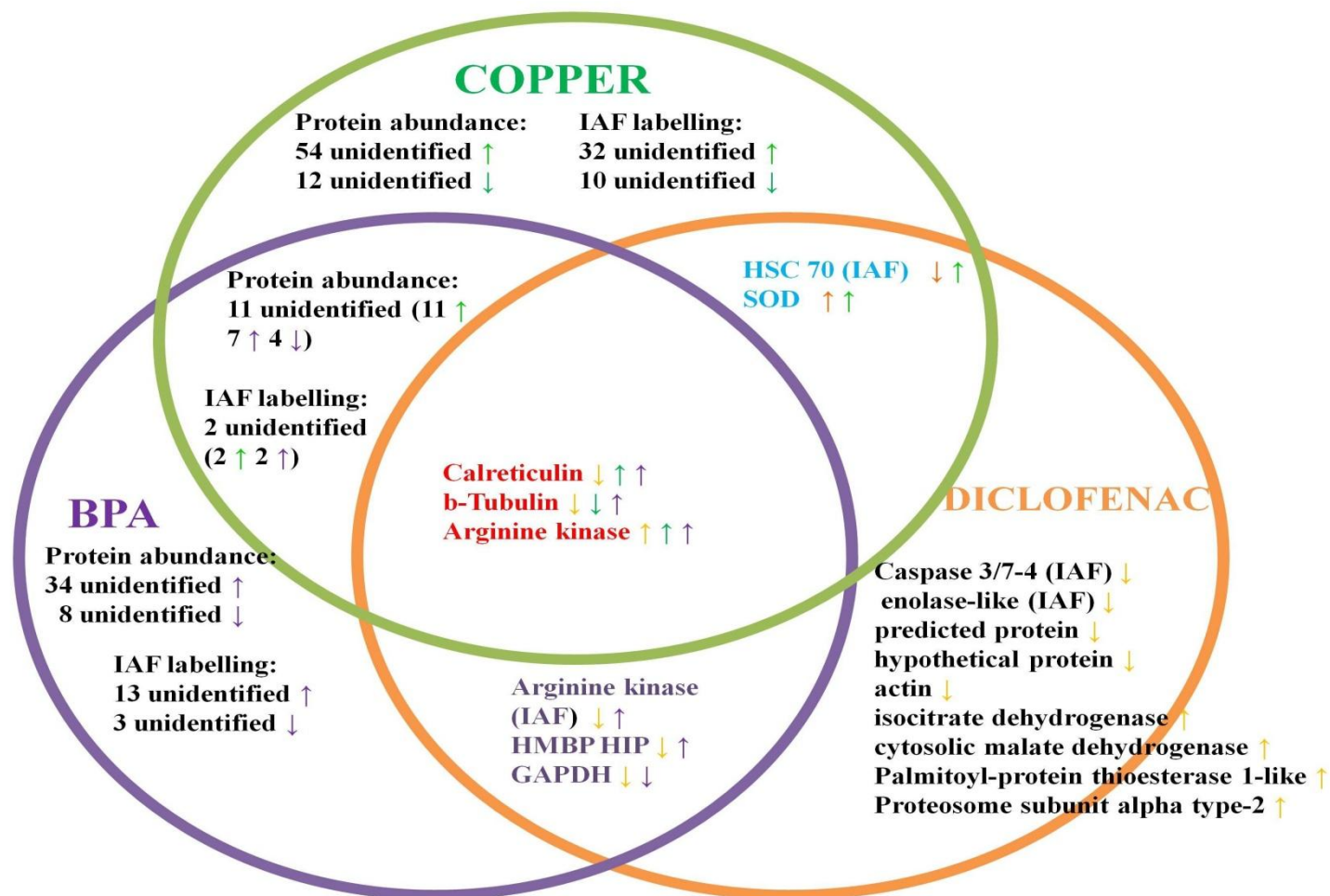


Figure 1. Venn diagram showed the summarise results of the protein identified from three different exposure to *M. edulis*; diclofenac, copper and bisphenol A.

6.3 Identification of proteins

MALDI-TOF/TOF facilitated identification of proteins based on SameSpots analysis of 2DE patterns obtained in response to diclofenac exposure. In total, sixteen proteins were identified of which six were hypothetical proteins, eight changed in abundance and four showed altered oxidation (Jaafar et al., 2015). The up- or down-regulation indicated that some of these proteins could be possible biomarkers of exposure to diclofenac. However, in this study, a spot-matching routine using SameSpots software facilitated extending these observations to other anthropogenic pollutants i.e. BPA and copper. Three proteins showed similarities across the 3 different chemicals; calreticulin, beta-tubulin and arginine kinase. Calreticulin occurs in the ER and is widely distributed in eukaryotic cells (Mendlovic, 2010). It is also found elsewhere in the cell including the cytoplasm, cell membrane, extracellular matrix (the protein scaffold found between cells) and is also involved in maintaining adequate calcium levels. Calreticulin also functions as a chaperone to help other proteins fold correctly (Mendlovic, 2010; Rodrigues et al., 2012). It has also previously been shown to be sensitive to thiol oxidation in *M. edulis* (McDonagh and Sheehan, 2007). Beta-tubulin is an important cytoskeletal protein previously reported in clam (*Ruditapes decussatus*), blue mussels (*Mytilus edulis*) and abalone (*Haliotis diversicolor supertexta*) after exposure to heavy metal and BPA (Chora et al, 2009; Hu et al., 2014; Zhou et al., 2010). This protein is involved in the structure and function of the cytoskeleton and a decrease in its level may result from oxidative stress effects (Chora et al., 2009; Miura et al., 2005). Beta tubulin was previously reported to be down-regulated in digestive gland of *Mytilus edulis* from polluted sites (Apraiz and Cristobal 2006).

Arginine kinase was the most consistent protein found across the 2DE patterns obtained in the three chemical exposures studied here. This enzyme is a member of the phosphagen kinase family which catalyzes the reversible transfer of a high-energy phosphate from the phosphagen arginine phosphate to ADP to form ATP (Holt & Kinsey, 2002). In posterior gills of the blue crab (*Callinectes sapidus*) and shore crab (*Carcinus maenas*) it has been shown that arginine kinase has significant enzymatic activity. The enzyme plays a key role as an ATP buffer in gill tissue (Kotlyar et al., 2000). Perhaps surprisingly, this enzyme has not previously been suggested as a putative biomarker in marine invertebrates. Mg: bb02e05 (Protein coding) is a variant of glyceraldehyde 3-phosphate dehydrogenase. This is a key redox sensor protein in energy metabolism (Hildebrandt et al., 2015). It has previously been reported to be significantly down-regulated in digestive gland of *Mytilus sp.* exposed to diclofenac and gemfibrozil (Schmidt et al., 2014). Oxidation of cytosolic hsc 70, was identified in both diclofenac and copper exposure. This suggested that diclofenac and copper induced cellular stress. During stress conditions, hsps move into the cell nucleus and begin to repair and protect nuclear proteins from aggregation leading to cell damage (Pint et al., 1991). HSP 70 was found in gills of *M. galloprovincialis* and *M. trossulus* in response to acute heat stress (Tomanek et al., 2010). Heavy metal-binding protein (HIP) is a common protein found in *M. edulis* gill (McDonagh and Sheehan, 2007; Fields et al., 2012; Rocher et al., 2015). HIP was previously isolated from hemolymph of *M. edulis* and binds heavy metals ions (Cd, Cu and Zn) thus contributing to their detoxification (Yin et al., 2005; Devoid et al., 2007). In the present study, HIP was found in both diclofenac and BPA exposure, but not in copper exposure. The principal function of these proteins in gills of *M. edulis* is probably in metal detoxification (Rocher et al.,

2015). Superoxide dismutase (SOD) was found to be significantly increased in diclofenac and copper exposure. SOD is one of the most important antioxidant defence enzymes in aerobic organisms and it was found to be significantly increased in gills and liver of carp (*Cyprinus carpio* exposed to diclofenac (Manduzio et al., 2003; Islas-Flores et al., 2013). It is known as a prominent antioxidant defence system that converts the superoxide anion into hydrogen peroxide and SOD is used widely as a biomarker of oxidative stress in ecotoxicology (Vlahogianni et al., 2007; Regoli & Principato, 1995).

6.4 Concluding remarks

This thesis presents a study of the use of traditional biomarkers of oxidative stress effects (antioxidant enzyme activity measurements) combined with proteomics methods to evaluate the effects of 3 different types of anthropogenic pollutants on the blue mussel, *M. edulis*; diclofenac, copper and bisphenol A. Throughout this investigation, redox proteomics methods were applied. Protein thiol oxidation was taken as indicative of level of general oxidative stress and was found to be variable across different experiments but quite responsive within experiments. IAF labelling of thiol-containing proteins revealed the redox status of protein cysteines using fluorescence detection. This approach leads to new potential biochemical endpoints which could be of relevance to ecotoxicology. Notably, calreticulin and arginine kinase are crucial to marine invertebrates and deserve greater study in the future. Mussels' gills tissues seem to be the main target for anthropogenic pollutants and gave a stronger oxidative response in all experiments. Study of oxidative stress using a combination of traditional biomarkers and redox proteomics methods has significant potential for the future in discovering new and robust biomarkers.

6.5 Future perspectives

Application of proteomics to environmental toxicology is still relatively undeveloped and is still bedevilled by under-representation of invertebrate genomes in sequence databases thus compromising protein identification (Dowling and Sheehan, 2006; Trapp et al., 2014). This is remarkable in an era of next-generation sequencing (Sheehan, 2013). However, the recent publication of an oyster genome (*Crassostrea gigas*) gives hope for the future (Zhang et al., 2012). Marine pollution assessment and monitoring of the effects of environmental stressors utilizing bivalves as sentinel organisms continues to be a priority research area in environmental science. Proteomic research in bivalves supported by high-throughput technologies is at the front line of the integration of genomics and proteomics data. This could improve biomonitoring programmes, help reveal mechanisms underlying pollutant toxicity and give insights into how organisms are adapting to climate change and habitat loss (Tomanek, 2011; Tomanek, 2012; Campos et al., 2012).

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APPENDIX