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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.

Keywords: Protein thiols; biomarkers; remediation; oxidative stress; proteomics; *Mytilus edulis*.

1. 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 km^2 [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 thi-

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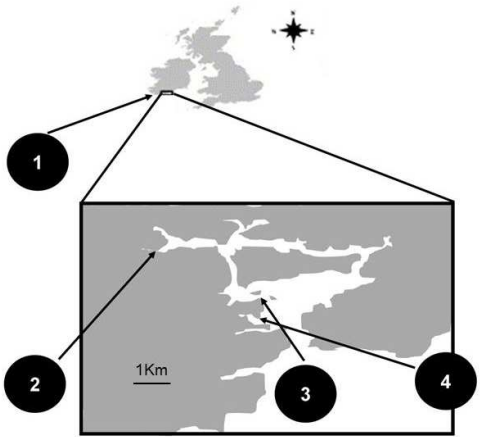

Figure 1. Sampling sites for *Mytilus edulis* around Cork harbour, Ireland: (1) Bantry Bay (out-harbour control site), (2) Douglas, (3) Haulbowline, (4) Ringaskiddy (in-harbour control site).

ols 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.

2. Materials and methods

2.1 *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 (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.

2.2 *Chemicals*

Acetonitrile, bovine serum albumin (BSA), 1-chloro-2,4-dinitrochlorobenzene (CDNB), dimethyl sulfoxide (DMSO), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 5'-iodoacetamide fluorescein (IAF), 1-methyl-2-phenylindole, neutral red, phenyl-methylsulphonylfluoride (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).

2.3 *Sample preparation*

Digestive glands were homogenized in a motor-driven Teflon Potter-Elvejhem 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].

2.4 *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.

2.5 *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 (H_2O_2). Activity was expressed as U/min/mg protein ε = -0.04 $mM^{-1}cm^{-1}$). 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 $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

2.6 *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 × *g* for 20 min and then derivatized in a 1 ml reaction mixture containing 10.3 mM 1-metyl-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 × *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].

2.7 *Labelling protein thiols*

Protein thiols present in protein extracts were labelled by adding IAF in DMSO to a final concentration of 800 µM 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].

2.8 *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 µl 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 2 h; 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].

2.9 *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.

2.10 *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 microcolumns using GELoader tips packed with POROS R2 (Applied Biosystems, Foster City, California, USA; 20 µm bead size) and then directly eluted onto the MALDI target plate using 0.5 µl 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), adrenocorticotropic 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.

2.11 *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 1x10-3) for additional information on functional pathway.

2.12 *Statistical analysis*

All data are means ± 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 $p<0.05$.

3. Results

3.1 *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).

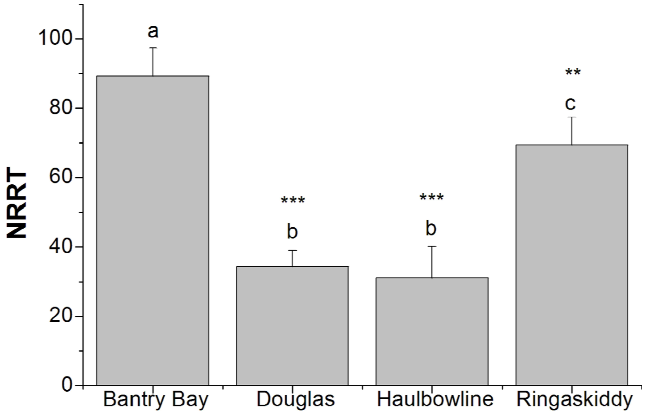

Figure 2. Lysosomal membrane stability measured as neutral red retention time (NRRT; min) in haemolymph from *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean ± SD. Superscripts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

3.2 *Antioxidant enzymes*

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

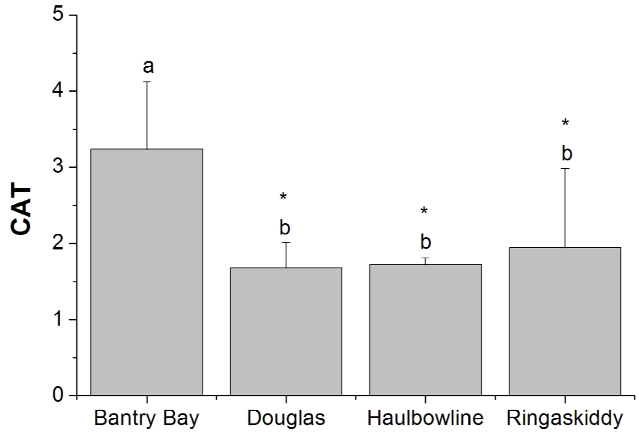

Figure 3. Catalase activities (CAT; U/min/mg prot) in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean ± SD. Superscripts of different letters are significantly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

3.3 *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).

3.4 *Protein thiols*

1DE separation of IAF-labelled proteins revealed a decrease in total thiol-containing proteins in samples from

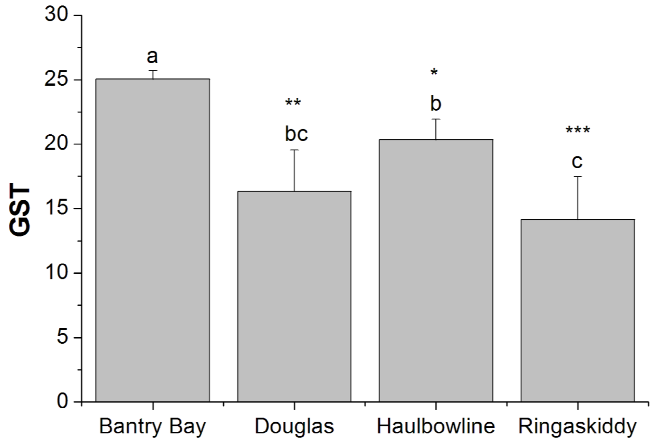

Figure 4. Glutathione transferase activities (GST; nmol CDNB conjugate formed/min/mg prot) in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean ± SD. Superscripts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

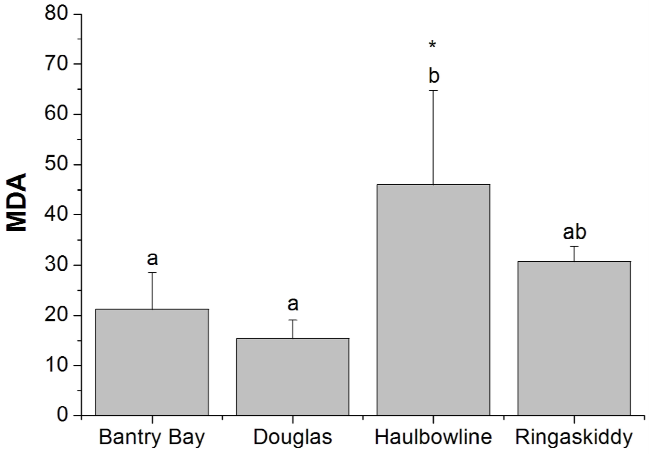

Figure 5. Lipid peroxidation measured as malondialdehyde (MDA) levels (nmol/g wet weight tissue) in digestive gland of *M.edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed as mean ± SD. Superscripts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

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.

3.5 *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).

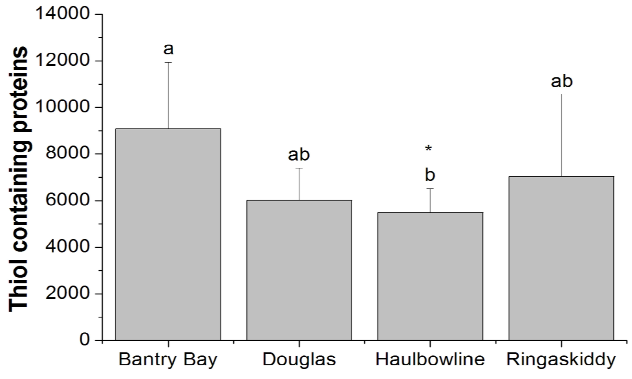

Figure 6. 1DE of thiol-containing proteins (normalized by total protein amounts) in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Data are expressed in A.U. as mean ± SD. Superscripts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

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).

4. 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. This was performed in this study and decreased IAF labeling is 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 Har-

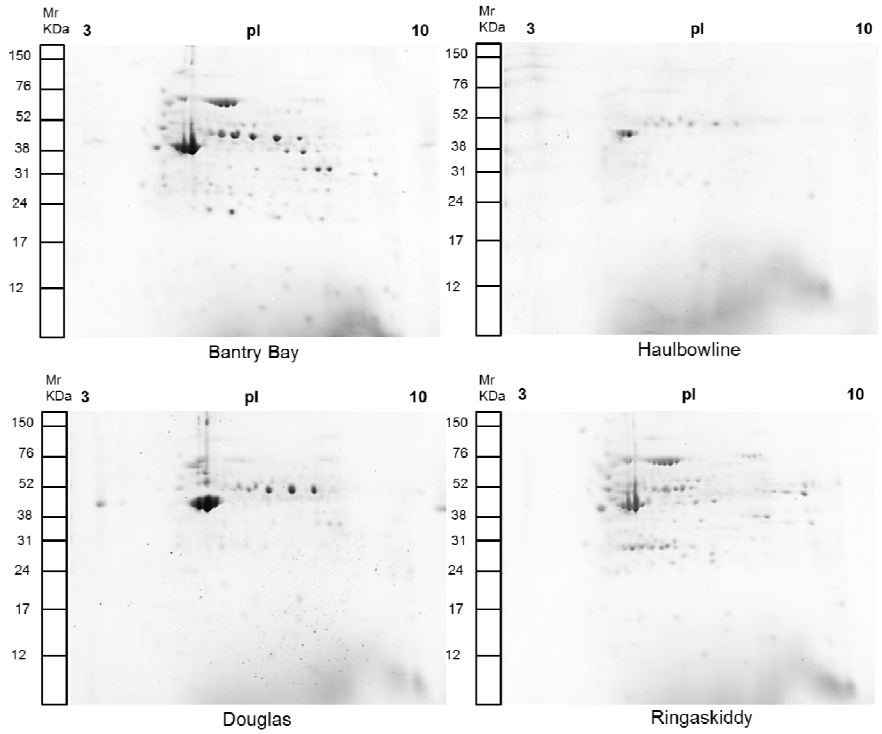

Figure 7. Representative 2DE of thiol containing proteins trapped by ATS in digestive gland of *M. edulis* collected in Bantry Bay (out-harbour control site), Douglas and Haulbowline (polluted sites) and Ringaskiddy (in-harbour control site). Spots present in Bantry Bay and/or Ringaskiddy 2DE but weakened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

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 identified	Function
1	gi|254553092	Endoglucanase	*Mizuhopecten yessoensis*	5,66/~5.5	64.152/52-76	120	1.5e-0.005	1	Carbohydrate metabolism
2	gi|301341836	Arginine kinase	*Conus novae-hollandiae*	6,34/~6.4	39.379/31-38	135	4.8e-007	1	Energy metabolism
3	gi|42627683	Creatine kinase 1	*Lethenteron camtschaticum*	6,71/~6.8	42.620/31-38	166	3.8e-010	1	Energy metabolism
4	gi|12230122	Endo-1,4-beta-glucanase	*Mytilus edulis*	6,79/~6	19.699/17-24	582	9.6e-052	5	Carbohydrate metabolism

bour, Ireland, were compared to in-harbour and out-harbour reference sites by measuring physiological and biochemical 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 slightly 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 cellulase 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.

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