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A proteomic analysis of the effects of metal contamination on Sydney Rock Oyster (*Saccostrea glomerata*) haemolymph

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ABSTRACT

The current study uses proteomics to assess the effects of metal contamination on Sydney Rock oyster haemolymph. *Saccostrea glomerata* were exposed in aquaria for four days to three environmentally relevant metals (copper, lead or zinc). Oyster haemolymph proteins from metal-exposed oysters were then compared to haemolymph from non-exposed controls using 2-dimensional electrophoresis to identify proteins that differed significantly in intensity. These proteins were then subjected to tandem mass spectrometry so that putative protein identities could be assigned. The data suggest that there are unique protein expression profiles for each metal. Exposure to 100 μg/l of copper, lead or zinc yielded a total of 25 differentially expressed proteins. However, only one of these protein spots exhibited altered intensities in response to all three metals. Eighteen of the 25 spots were significantly affected by just one of the three metals. Differentially expressed proteins were assigned to five different categories of biological function. Proteins affecting shell properties were the most common functional group accounting for 34% of the identified proteins. Cytoskeletal activities and metabolism/stress responses each accounted for a further 25% of the proteins.

1. Introduction

The introduction of waste products into rivers and estuaries, especially those in industrial and urbanized areas, has led to a significant increase in chemical contamination of coastal environments worldwide (Li et al., 2000). Heavy metals and trace metals are naturally occurring but have increased in the environment, especially urban waterways, due to anthropogenic exploitation for use in industrial and manufacturing processes (Taylor et al., 2004). Substantial evidence suggests that such metal contamination has the potential to induce significant changes in biota and ecosystems (Hardiman and Pearson, 1995).

Australia is one of the most urbanized western nations in the world with the majority of its population living in close proximity to a few highly urbanized coastal estuaries or embayments including Port Jackson (Sydney), Port Melbourne and Moreton Bay (Brisbane) (Australian Bureau of Statistics, 2010). This raises particular concerns for the potential degradation of these ecosystems and their associated biota. Threats from metal inputs means that a range of effective methods are required to identify, monitor and remediate such contaminants.

At present there are three broadly accepted methods for environmental monitoring: chemical analysis, ecotoxicology and the use of molecular biomarkers. Chemical analyses, such as sediment toxicity and water quality analyses, have provided useful mapping information of contaminants in sediments and water (Li et al., 2000; Hatje et al., 2001; Roach, 2005). However, these analyses provide no indication of the impact of contaminants on biota. Ecotoxicology, whilst ecologically and biologically relevant, may lack the sensitivity to discriminate between different contaminants or to detect sub-acute physiological changes before permanent damage occurs (Peter et al., 1998; Regoli et al., 2002).

The limitations of these methods mean that additional technologies are required to differentiate between a variety of contaminants, distinguish between transient and long-term biological damage, and provide sufficient sensitivity to allow early warning of environmental damage. Proteomics has the potential to fulfil these requirements and become part of a weight of evidence approach integrating a range of monitoring techniques. Proteomics allows the simultaneous analysis of hundreds of potential molecular markers and provides information on the

Abbreviations: Cd, cadmium; $Cu/CuCl_2$, copper chloride; $Pb/PbCl_2$, lead chloride; $Zn/ZnCl_2$, zinc chloride; 2-DE, 2-dimensional electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; DTT, DT dithiothreitol; IAA, iodoacetamide; IEF, isoelectric focussing; SDS, sodium dodecyl sulfate; MS, mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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effects of contaminants on a broad range of biological functions (Dhingra et al., 2005). Proteomic analyses have already revealed clear relationships between protein concentrations and environmental stress (Gomiero et al., 2006). This suggests that proteomics has the potential to be a robust biomonitoring technique (Viant et al., 2002; Jonsson et al., 2006) providing unique protein expression signatures for anthropogenic chemical contamination (Bradley et al., 2002). Although proteomics has also been used to monitor contaminant effects in the natural environment (Montes Nieto et al., 2010; Cajaraville et al., 2000; Knigge et al., 2004) it can be a time consuming and expensive technique. Once proteins have been identified from these laboratory-based proteomic studies, antibodies to those proteins will be used in ELISAs to enable the fast and inexpensive analysis of field samples.

The current study uses proteomics to analyse changes in the proteomes of haemolymph from the Sydney Rock oyster, *Saccostrea glomerata*, after exposure to copper (Cu), lead (Pb) and zinc (Zn) under controlled laboratory conditions. Bivalve molluscs, such as *S. glomerata*, have been used extensively in biomonitoring due to their efficiency in bio-accumulating contaminants and their ability to show time-and dose-dependent relationships to contaminant exposure (Tanabe et al., 2000; Chase et al., 2001; Gillikin et al., 2005). *S. glomerata* are ubiquitous and ecologically relevant in estuaries along the east coast of Australia. The ability of this species to bio-accumulate contaminants rapidly and depurate them slowly makes them an ideal test species for the current study (Brown and McPherson, 1992; Hardiman and Pearson, 1995; Scanes, 1997; Scanes and Roach, 1999). Similarly, bivalve haemolymph has been used to study the effects of exposure to polycyclic-aromatic hydrocarbons (PAHs) (Grundy et al., 1996), and metals (Shepard and Bradley, 2000). The current study analyses the effects of 100 μg/l doses of Cu, Pb or Zn, which lie within the limits of concentrations used in other work assessing cellular responses of metal exposure on bivalves, for example, 0.1 μg/l to 80 μg/l Cu in *Crassostrea virginica* (Ringwood et al., 1998); 180 μg/l Pb in *Pinctada imbricata* (MacFarlane et al., 2006); 10 to 100 μg/l Zn in *Perna viridis* (Shi and Wang, 2004). The concentrations of metals used here also represent environmentally relevant doses that occur in the field (Birch and Olmos, 2008) and are based on Australian and New Zealand Environment and Conservation Council sediment and water quality guidelines (ANZECC/ARMCANZ, 2000).

2. Materials and methods

2.1. Oyster acclimation and exposure to metals

For this laboratory-based exposure trial, *S. glomerata* were purchased from Aquaculture Enterprises (Eden, NSW). They were between 18 months and 2 years old and were from the same tray-reared hatchery spat. Oysters were exposed to metals in 12 × 25 l aquaria at the Sydney Institute of Marine Science (Chowder Bay, Sydney, Australia). Ten oysters were placed in each aquarium for 10 days prior to metal exposures to acclimate them to aquarium conditions. Complete water changes using seawater pumped directly from Port Jackson were performed daily during the trial and oysters were fed every four days with M-1 bivalve feed (Aquasonic, Wauchope, NSW). Water temperature and salinity were recorded daily. Water samples were taken prior to the start of the study and tested for a suite of metals, PCBs and PAHs.

Following the 10 day acclimation period (preliminary studies in our laboratory suggest it takes 10 days for oysters to acclimate to aquaria conditions before controlled stress-response experiments can be successfully conducted), oysters were exposed separately for four days to 100 μg/l $CuCl_2$, 100 μg/l $ZnCl_2$ or 100 μg/l $PbCl_2$. Each metal was placed in a set of three aquaria with the remaining three aquaria used as controls with no added metals. The water in each aquarium was changed daily with the same concentrations of metals. Stock solutions of metals were dissolved in seawater before being added to aquaria.

2.2. Protein extraction

After the four day exposure period, oysters were shucked and 500 μl of haemolymph was harvested per oyster from the pericardial cavity using a micropipettor. The haemolymph was immediately mixed within 1.3 ml of Tri-reagent LS (Sigma–Aldrich) to lyse haemocytes. RNA was removed by adding 100 μl of bromochloropropane for 15 min followed by centrifugation for 15 min at 12,000 × *g* (4 °C) and removal of the colourless aqueous phase. DNA was then extracted by adding 300 μl of 100% ethanol for 3 min followed by centrifugation at 2000 × *g* for 5 min (4 °C) and discarding of pellet. Finally, proteins were precipitated by adding 3 volumes of ice cold acetone, standing samples at room temperature for 10 min and centrifugation for 10 min at 12,000 × *g* (4 °C). The remaining protein pellet was then washed four times for 10 min per wash in 1 ml of 0.3 M guanidine hydrochloride in 95% ethanol (V:V) followed by centrifugation at 8000 × *g* for 5 min (4 °C) and removal of the supernatant. A final wash was performed in 1 ml of 95% ethanol before protein pellets were dried at room temperature and re-suspended in 50 μl of re-hydration buffer (7 M urea: 2 M thiourea: 4% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, CHAPS: 50 mM DT dithiothreitol, DTT).

2.3. Protein quantification and pooling of samples

Proteins were quantified using GE 2DE Quant Kits with protocols modified from manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Two microlitres of each sample was added to each of three wells of 96-well microtiter plates followed by 10 μl of copper solution, 40 μl of Milli Q water and 100 μl of colour reagent. After incubation at room temperature for 20 min, absorbance was measured at 490 nm and protein concentrations were interpolated from a standard curve generated with bovine serum albumin. Five randomly selected haemolymph samples from each aquarium were pooled. Ten oysters were placed in each aquarium to ensure enough oysters were available for proteomic analysis after any mortality but five oysters were sufficient to provide enough protein for analysis. A total of three replicates of pooled samples were prepared per treatment plus three replicates of non-exposed controls. Each pooled replicate contained 150 μg of protein.

2.4. 2-Dimensional electrophoresis (2DE)

Isoelectrofocusing (IEF) was performed using an IPGphor IEF system (GE Healthcare). Immobilized pH linear gradient (IPG) gel strips (7 cm, pH 4–7; GE Healthcare) were re-hydrated overnight with 150 μg of extracted proteins (as described above) in 125 μl rehydration buffer (7 M urea: 2 M thiourea: 4% CHAPS: 50 mM DTT and 0.5% carrier ampholytes, GE Healthcare). IEF was performed at 100 V for 2 h, 500 V for 20 min, a gradient up to 5000 V for 2 h and 5000 V for 2 h to give a total of 15,000–16,000 voltage hours. The IPG strips were reduced (1% DTT, 20 min) and alkylated (2.5% iodoacetamide, 20 min) before second dimension separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12% Tris–HCl polyacrylamide gels (1.5 M Tris–HCl: 10% SDS: 12% acrylamide) in a Mini PROTEAN system (Bio-Rad, CA, USA). Gels were stained using Lava Purple (Fluorotechnics, Sydney, Australia) and visualised using a Typhoon Trio laser scanner (GE Healthcare).

2.5. Protein spot analysis

2DE proteome maps were analysed to identify protein spots which differed significantly in fluorescence intensity between control gels and metal-exposed treatments using Progenesis proteomic analysis software (Non Linear Dynamics, Newcastle-upon-Tyne, UK). The three 2DE gels per metal treatment (each representing pooled protein samples from a single aquaria) were compared against the three control (non-exposed) gels.

Progenesis was used to perform Students *t*-tests on the fluorescent intensity values for each spot. Those with p-values < 0.05 were considered to be significantly differentially expressed. Fold differences for each differential spot were calculated from the mean normalized volumes (average spot intensity from each gel normalized to background intensity of the gel) of controls compared to the respective metal treatment. A positive fold value represents an increase in spot intensity in the treatment compared to the control, whilst and a negative fold value represents a decrease in spot intensity in the metal treatment compared to the control.

2.6. Peptide extraction

Once spots with differential intensities were identified a new set of 2DE gels were prepared and stained with Coomassie silver blue (Candiano et al., 2004) to facilitate spot extraction. Gel plugs containing protein spots from all three gels for each treatment were washed briefly with 100 mM ammonium bicarbonate (NH_4HCO_3) before destaining in 50:50 acetonitrile (ACN)/50 mM NH_4HCO_3 (three washes for 10 min per wash). After dehydration in 100% ACN for 5 min, gel plugs were air dried, reduced with 100 mM DTT in 100 mM NH_4HCO_3 at 56 °C for 1 h and alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 min at room temperature in the dark. The gel plugs were again washed in 100 mM NH_4HCO_3 for 5 min then twice with 50:50 ACN/50 mM NH_4HCO_3 for 5 min and dehydrated as described above. Trypsin digestion was undertaken by adding 30 μl trypsin solution (12.5ng/μl in 50 mM NH_4HCO_3, Promega, Sydney, Australia) for 30 min at 4 °C, after which proteins were digested overnight at 37 °C. Tryptic peptides were extracted by washing gel plugs twice in 50% ACN/2% formic acid for 30 min. The resulting supernatants were reduced to 12 μl in a vacuum centrifuge and further centrifuged for 10 min at 14,000 rpm to remove microparticles.

2.7. Mass spectrometry

Tryptic peptides from excised spots were analysed by nanoflow liquid chromatography–tandem mass spectrometry (LC–MS/MS) using an LTQ-XL ion-trap mass spectrometer (Thermo, CA, USA) according to Breci et al. (2005). Reversed phase columns were packed in-house to approximately 7 cm (100 μm i.d.) using 100 Å, 5 mM Zorbax C18 resin (Agilent Technologies, CA, USA) in a fused silica capillary with an integrated electrospray tip. A 1.8 kV electrospray voltage was applied via a liquid junction up-stream of the C18 column. Samples were injected onto the C18 column using a Surveyor autosampler (Thermo, CA, USA). Each sample was loaded onto the C18 column followed by an initial wash step with buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) for 10 min at 1 μl min^{-1}. Peptides were subsequently eluted from the C18 column with 0%–50% Buffer B (95% (v/v) ACN, 0.1% (v/v) formic acid) over 58 min at 500 nl min^{-1}, followed by 50%–95% Buffer B over 5 min at 500 nl min^{-1}. The column eluate was directed into a nanospray ionization source of the mass spectrometer. Spectra were scanned over the range 400–1500 amu. Automated peak recognition, dynamic exclusion, and tandem MS of the top six most intense precursor ions at 35% normalization collision energy were performed using Xcalibur software (version 2.06) (Thermo, CA, USA). Raw data files were converted to mzXML format and processed through the Global Proteome Machine (GPM) software using version 2.1.1 of the X!Tandem algorithm, freely available from www.thegpm.org (Craig and Beavis, 2003; Craig and Beavis, 2004).

2.8. Protein identification

MS/MS spectra were searched against a *Bivalvia* database created with sequences downloaded from the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). This FASTA format database contained 14,002 protein sequences comprising actual or predicted protein sequences for bivalve molluscs held by NCBI as of August 2009. The database also incorporated a list of common peptide contaminants. A search was also performed against a reversed sequence database to evaluate the false discovery rate (FDR). Search parameters included MS and MS/MS tolerances of ±2 Da and ±0.4 Da, tolerance of up to 3 missed tryptic cleavages and K/R-P cleavages. Fixed modifications were set for carbamidomethylation of cysteine and variable modifications were set for oxidation of methionine. Only proteins that had $log(e)^+$ values of <−10 and yielded at least five matching peptides were retained for further analysis. Using these criteria, no non-palindromic reverse database protein identifications were detected. Raw data files were also converted to the Mascot generic format (mgf) and searched against the NCBI (non-redundant) database using the Mascot MS/MS ion search platform (www.matrixscience.com) to confirm identifications and cross reference results. Search parameters included MS and MS/MS tolerances of ±1.4 Da and ±0.6 Da, tolerance of up to 1 missed tryptic cleavages. Fixed modifications were set for carbamidomethylation of cysteine and variable modifications were set for oxidation of methionine. Proteins were assigned a biological function based on their functional annotations in the NCBI database and in published literature.

3. Results

3.1. Water quality parameters

The average water temperature throughout the study was 19.8 °C. Significant daily differences ($p < 0.05$) in water temperature were found on three days between aquaria containing Zn exposed oysters compared to all other treatments, however, the difference was only up to 0.7 °C lower in Zn aquaria than other aquaria. Salinity averaged 34.5 ppt and no significant differences were found between treatments or control aquaria throughout the period of study. Levels of metals in water samples were all at <2 μg/l, PCBs <0.1 μg/l and PAHs <2 μg/l.

3.2. Protein expression patterns after exposure to different metals

Progenesis software identified an average of 161 distinct protein spots per oyster haemocyte proteome (2DE gel). These spots were distributed predominantly in the pI range of 4–7. A typical proteome map from this study is shown in Fig. 1A. Fig. 1B and C highlight spots 239 and 270 respectively showing the differences in average normalized intensities of these two spots compared to controls. Of the 161 spots, the relative intensities of 25 spots across all treatments varied significantly ($p < 0.05$) after metal exposure. Four of these 25 spots changed in response to more than one metal, so that there were a total of 21 unique spots detected in the entire analysis. Fig. 2 shows the number of spots that responded to each metal exposure, and where crossovers occurred if a protein spot responded to more than one metal exposure.

Only one protein spot (spot 299) exhibited significantly different intensities in oysters from all three metal treatments relative to controls. When exposed to Cu two additional spots (spots 303

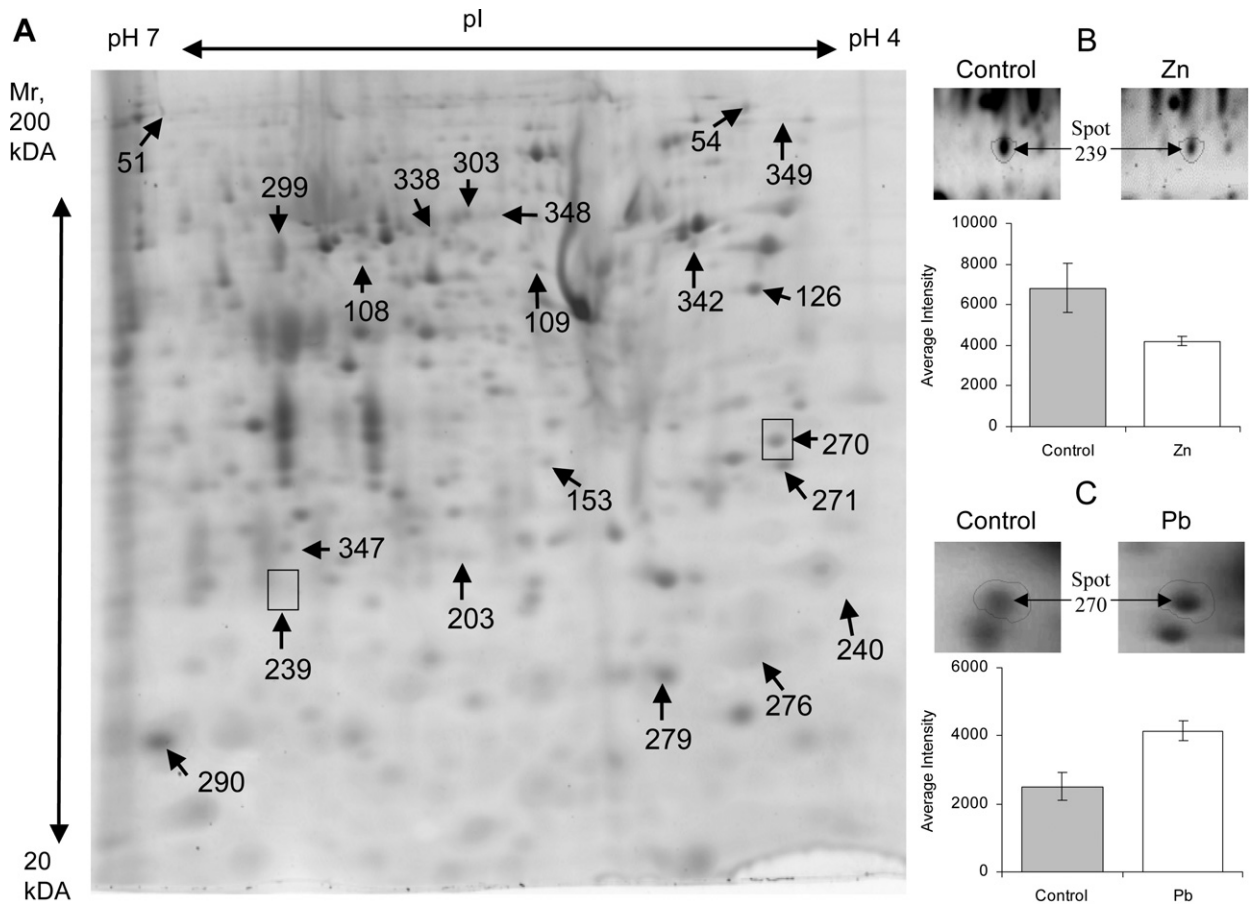

Fig. 1. A. A 2DE gel from the study showing the location of protein spots that exhibited significantly different intensities ($p < 0.05$) compared to controls when oysters were exposed to 100 μg/l Cu, Pb, or Zn. Spot numbers were assigned arbitrarily by Progenesis software. The boxed areas are magnified in panels B and C which show the average intensities of two spots that significantly changed in intensity ($n = 3$, bars – SEM). Mr – molecular weight, pI – isoelectric point. B. Differentially expressed spot number 239 in control versus Zn exposed gels. C. Differentially expressed spot number 270 in control versus Pb exposed gels.

and 348) showed statistically significant changes compared to controls ($p < 0.05$). Exposure to Pb yielded the greatest number changes in spot intensities with 12 spots varying significantly ($p < 0.05$) in intensities from controls. Of these, nine responded to Pb only (spots 51, 54, 126, 153, 240, 270, 338, 342, 347) and two were significantly altered ($p < 0.05$) by Pb and Zn (spots 108 and 109). Ten spots showed significantly different intensities in response to Zn. Of these, seven were altered significantly ($p < 0.05$) in response to just Zn (spots 203, 239, 271, 276, 279, 290 and 349).

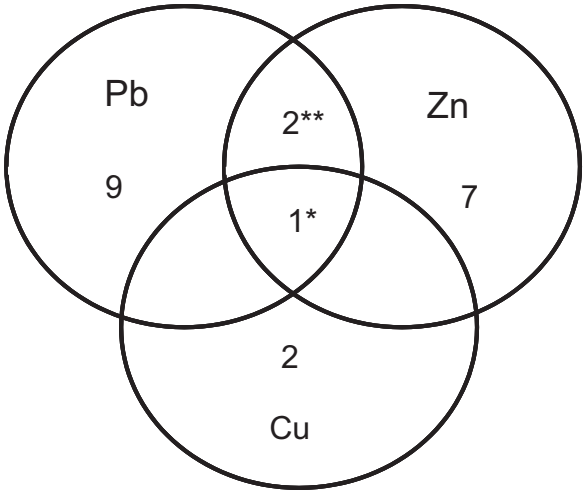

Fig. 2. The numbers of protein spots that differed significantly ($p < 0.05$) in intensity between oysters exposed to metals compared to non-exposed controls. *One spot changed in response to all three metal exposures, **two spots changed in response after exposure to two metals.

Fourteen of the 25 differential spots increased in intensity after metal exposure when compared to controls, whilst the remainder decreased in intensity. All of the spots that showed significant changes in response to Cu exposure decreased in intensity relative to controls. In contrast Pb exposure yielded ten spots that increased in intensity and only two that decreased. In Zn exposed oysters, 6 out of 10 spots decreased in intensity relative to controls.

Fig. 3 and Table 1 show that proteins from metal-exposed oysters differed in intensity by between 1.1 and 46.0 fold when compared to controls. The highest fold change was in spot 299, which decreased in intensity relative to controls by 46, 38 and 40 fold in oysters exposed to Cu, Pb and Zn respectively.

3.3. Protein identification

Of the 21 different protein spots 20 were able to be picked for mass spectrometric analysis. Of these 20 spots, 18 were putatively identified by comparison to sequences in the custom *Bivalvia* sequence database or the non-redundant protein sequence database at the NCBI. Five spots (spots 338, 342, 203, 276 and 108) contained peptides that were closely related to more than one protein in these databases. Details of putatively identified spots are shown in Table 2.

Table 1
Average normalized spot volumes (intensities) of three control gels compared to average normalized spot volumes of three gels from each metal exposure showing *p* values for comparisons between metal exposures and controls, and fold differences in intensity (normalized spot volume) relative to controls.

Metal exposure	Spot	Averaged normalised volumes		*T*-test (*p* value)	Fold change
		Control	Metal		
Cu	299	13408	288	0.005	−46.0
	303	7212.47	3607.313	0.001	−2.0
	348	2261.706	711.41	0.038	−1.8
Pb	51	539	2851	0.004	+5.2
	54	539	2483	0.02	+4.6
	108	1700	349	0.03	−4.8
	109	539	4121	0.006	+7.7
	126	1171	6683	0.02	+3.8
	153	2410	9882	0.04	+4.0
	240	308.267	848.485	0.001	+2.8
	270	2501.835	4195.417	0.049	+1.7
	299	13408	349	0.005	−38.0
	338	19150	21010	0.048	+1.1
	342	9614.993	19300	0.005	+2.0
	347	7012.352	16460	0.01	+2.3
Zn	108	1700	4608	0.01	+2.7
	109	539	4227	0.006	+7.7
	203	1105.801	1605.81	0.037	+1.5
	239	6822.119	4232.631	0.023	+1.6
	271	45180	38250	0.014	+1.2
	276	2394	4297.5	0.041	+1.8
	279	221800	129200	0.02	+1.7
	290	199700	157800	0.033	+1.3
	299	13408	333	0.004	−40.0
	349	2750	333	0.01	−8.2

+ denotes an increase in intensity relative to controls and − denotes a decrease in intensity.

The characterized proteins (excluding those from spots that returned more than one putative identification), were assigned to five different functional categories. The attribution of these biological functions is shown in Figure 4. Proteins affecting shell properties were the most common functional group accounting for 34% of the identified proteins. Their putative functions included shell adhesion and shell calcification. All of these proteins were associated with Zn exposure. Cytoskeletal activities and metabolism/stress response comprised a further 25% of the proteins each, with RNA metabolism and cell signaling each accounting for 8%. Differential proteins associated with Pb exposure showed the broadest range of biological functions, including energy metabolism, stress response, cell signaling and cytoskeletal activities.

4. Discussion

We have identified significant changes in the proteome of oyster haemolymph after exposure to three metals commonly found in the aquatic environment. There was relatively little similarity between the patterns of the 21 different protein spots affected by the metals, with only one spot (spot 299) affected after exposure to all three metals.

Proteins in 18 of the 21 different spots were definitively identified by MS. Peptides from spots 51, 270 and 348 could not be matched to known sequences in the database used in this study. There is relatively little sequence data available for *S. glomerata* and so it was not unexpected that some proteins could not be identified.

Five proteins (spots 108, 203, 276, 338 and 342) contained peptides that matched more than one sequence in the databases. It is not uncommon for more than one distinct protein to occupy the same or very similar locations on 2DE gels. Whilst several proteins in spots that contained more than one protein have been previously associated with environmental stress (Kostrzewska and Sobieszek, 1990; Nicholson and Lam, 2005; Silverman and Roberto, 2007), we cannot say unequivocally that these proteins accounted for the differential intensities of the spots in which they were detected during the current study.

Of the spots that contained more than one protein, spot 108 changed in intensity in response to both Pb and Zn. It contained

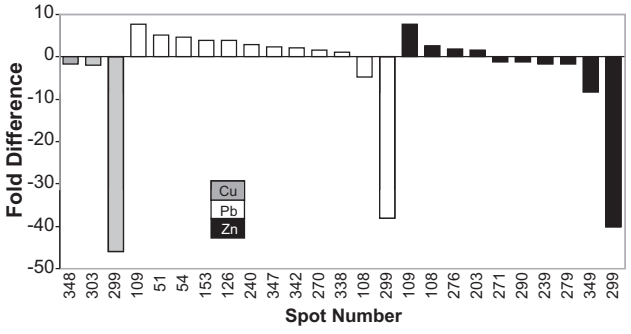

Fig. 3. Fold differences relative to non-exposed controls of the 25 protein spots that differed significantly in intensity between metal-exposed oysters and non-exposed controls. *n* = 3.

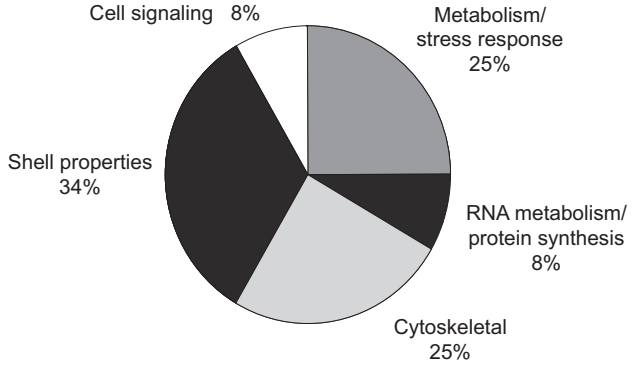

Fig. 4. Ontology of putative biological functions assigned to protein spots that were found at significantly different intensities in response to 100 μg/l Cu, Pb or Zn relative to controls.

Table 2
Identification of proteins in 2DE gel spots that differed significantly in intensity between metal-exposed and non-exposed oysters.

Metal	Spot #	# of peptides	log(e)$^+$ value	Mr	Homology to protein (ncbi accession number and species name)	Putitative protein identification	Annotated biological function
Cu	303	5	−39.2	53.6	gi|9957079| [*Placopecten magellanicus*]	Omega-crystallin	Energy metabolism and stress response
	348					Unable to identify	
Pb	51					Unable to identify	
	54	5	−39.9	18.9	gi|158515777| [*Saccostrea glomerata*]	Vitellogenin	Lipid transport/oxidative stress
	126	22	−223.2	33.5	gi|229891605| (*Pinctada fucata*)	40S ribosomal protein	Protein synthesis
	153	5	−39.9	33	gi|15419048| [*Crassostrea gigas*]	Tropomyosin	Cytoskeletal
	240	17	−46.1	18.2	gi|40642994| [*Crassostrea gigas*]	Myosin essential light chain	Muscle protein or cytoskeletal
	270	5	−30.3	50.9		Unable to identify	
	338	6	−55.2	49.9	gi|56603670| [*Crassostrea gigas*]	Beta-tubulin	Cytoskeletal
		5	−41.9	53.6	gi|9957079| [*Placopecten magellanicus*]	Omega-crystallin	Energy metabolism and stress response
	342	37	−412.1	56.6	gi|86156234| [*Pinctada fucata*]	ATP synthase beta subunit	Energy metabolism
		28	−393.5	49.9	gi|194068375| [*Saccostrea kegaki*]	Beta-tubulin	Cytoskeletal
		6	−56	96.6	gi|56603668| [*Crassostrea gigas*]	Alpha-tubulin	Cytoskeletal
	347	16	−175.7	16.2	gi|46909461| [*Mytilus edulis*]	Triosephosphate isomerase	Energy metabolism
Zn	203	6	−39	42.5	gi|13537551| [*Mizuhopecten yessoensis*]	Myosin	Cytoskeletal
		5	−33.7	223	gi|124055243| [*Mizuhopecten yessoensis*]	Polyprotein	DNA integration and replication
		5	−30	28.3	gi|3513512| [*Mytilus edulis*]	Non-gradient byssal precursor	Shell adhesion
	239	10	−66.2	28.3	gi|3513512| [*Mytilus edulis*]	Non-gradient byssal precursor	Shell adhesion
	271	16	−116.3	28.3	gi|3513512| [*Mytilus edulis*]	Non-gradient byssal precursor	Shell adhesion
	276	5	−30.7	151.7	gi|124055243| [*Mizuhopecten yessoensis*]	Polyprotein	DNA integration and replication
		6	−42.1		gi|21105303| [*Mytilus galloprovincialis*]	Precollagen-D	Shell adhesion
	279	28	−219.2	61.7	gi|2204081| [*Pinctada fucata*]	Insoluble protein	Shell calcification
	290	7	−48.9	28.4	gi|93102311| [*Pinctada fucata*]	Shematrin-7	Shell calcification
Pb/Zn	108	10	−73	28.3	gi|3513512| [*Mytilus edulis*]	Non-gradient byssal precursor	Shell adhesion
		5	−30.9	110.3	gi|152003987| [*Pinctada fucata*]	Sarco/endoplasmic reticulum calcium ATPase isoform C	Calcium ion transport
	109	31	−402.2	41.8	gi|3182893| [*Crassostrea gigas*]	Actin	Cytoskeletal
Cu/Pb/Zn	299	6	−38.4	82.7	gi|91179150| [*Chlamys farreri*]	Vasa	RNA metabolism

Putative identifications were accepted if the number of peptides identified per protein was ≥5 and the log(e)$^+$ value was ≤ −10. Mr – theoretical molecular weight.

non-gradient byssal precursor and sarco/endoplasmic reticulum Ca^{2+} ATPase isoform C (SERCA). Non-gradient byssal precursors are glycine-rich proteins with adhesive properties linked to byssal threads. Their expression in *M. edulis* is affected by biotic and abiotic factors including altered salinity, substrate type and stress (Silverman and Roberto, 2007). SERCA is a membrane protein which coordinates Ca^{2+} ion fluxes from the cytoplasm that in turn regulates cellular functions such as cell proliferation, apoptosis, contraction and metabolism (Nagata et al., 1998; Hoon Cho et al., 2000; Martin et al., 2002). Spots 203 and 276 changed in intensity in response to Zn exposure. Spot 203 contained non-gradient byssal precursor (as described above), myosin and polyprotein. Myosin is a motor protein associated with cell motility and organelle transport (Sellers, 2000). Polyprotein is responsible for DNA integration and RNA dependent DNA replication in *Chlamys farreri* (Wang et al., 2008). Polyprotein was also identified as being present in spot 276 along with pre-collagen D. Pre-collagen D assists in determining the structure of the byssus in *M. edulis* and *M. gallioprovincialis* (Vaccaro and Waite, 2001; Lucas et al., 2002). Spots 342 and 338 both significantly changed in intensity in response to Pb exposure. ATP synthase and alpha- and beta-tubulins were identified in spot 342. The enzyme complexes of ATP synthase contribute to cellular energy provision (Leyva et al., 2003). Alpha- and beta-tubulin make up part of the cytoskeleton and are important in maintaining shape and support for cells (Alberts et al., 2002). Apraiz et al. (2006) suggest beta-tubulin is one of the first targets of oxidative stress. Beta-tubulin and omega-crystallin were both inferred from spot 338. Omega-crystallin is a multi-functional enzyme associated with physiological stress (Piatigorsky et al., 2000).

Twelve of the protein spots that differed in intensity between metal exposures and controls were found to contain a single protein. Among these, molecules involved in shell adhesion or calcification accounted for 34% of the proteins exhibiting differential intensities, whilst cytoskeletal proteins and energy metabolism/stress response molecules each accounted for 25%. Of the remaining two proteins, vasa is involved in RNA metabolism and 40S ribosomal protein SA in cell signalling (or protein synthesis).

The vasa homologue exhibited by far the largest fold differences in intensity relative to controls, decreasing by between 38 and 46 fold in response to the three metals. It was also the only protein affected by exposure to all three metals. Vasa is a member of the DEAD box helicase family of proteins. It is associated with scaffolding during RNA metabolism in the mussel *M. gallioprovincialis,* with the potential to alter the structure of secondary RNA (Venier et al., 2006) and affect mRNA translation. Hence, the changes seen in the concentrations of vasa after exposure to metals could significantly impact on the synthesis of proteins required by *S. glomerata* to respond to metal induced stress. The large fold differences seen in vasa in response to all metals suggest that this protein could be used in biomonitoring as an indicator for a range of metal contaminants.

40S ribosomal protein SA, also known as laminin receptor 1, is involved in many cellular processes including cell adhesion to extracellular matrices, which affects signaling, differentiation and migration (Mecham, 1991). In this context Zn is known to affect the capacity of haemocytes in *M. gallioprovincialis* to attach to laminin suggesting a signaling role in cell growth and migration (Kaloyianni et al., 2006). Our study suggests Pb also changes laminin receptor intensity with potential to impact on these cellular processes.

Actin increased in concentration by 7.7 fold in response to both Pb and Zn exposures. It is a key cytoskeletal protein that is known to have roles in movement, phagocytosis, endocytosis, exocytosis, vesicular transport and cellular plasticity (Reisler, 1993; Cadoret et al., 1999; Miyamoto et al., 2002; Gagnaire et al., 2007). Whilst the disorganization of actin and its impact on cytoskeletal functions has been specifically linked to Cu exposure (Fagotti et al., 1996) in *M. gallioprovincialis*, the current study links the impact on actin's role in cytoskeletal activity to Pb and Zn. Two other key components of the actin cytoskeleton, myosin and tropomyosin, were also affected. Pb exposure led to a four-fold increase in the concentration of tropomyosin and a 2.8-fold increase in myosin essential light chain. Myosin is a motor protein responsible for actin-based motility (Sellers, 2000). Metals, such as cadmium (Cd), are known to affect myosin phosphorylation, altering its function (Kostrzewska and Sobieszek, 1990). Tropomyosin is an actin-binding protein important for regulating motile and contractile functions (Bailey, 1957). These combined effects of metal exposure on three important cytoskeletal molecules in *S. glomerata* are likely to affect the motility and cellular plasticity of haemocytes which are associated with their anti-pathogen activity of these cells. This may leave the oysters more susceptible to infectious disease. Our data suggest that Pb exposure has the most substantial effects to cytoskeletal associated proteins.

Three molecules involved in shell adhesion or calcification (non-gradient byssal precursor, insoluble protein and shematrin-7) were affected by exposing oysters to Zn but not by the other metals. The concentrations of all three of these proteins decreased by between 1.2- and 1.7-fold after exposure to Zn. Non-gradient byssal precursor proteins have adhesive properties. Studies on the mussel *P. viridis*, have shown that the byssus is affected by environmental pollution and that Cu inhibits byssal formation (Nicholson and Lam, 2005). Byssal threads in oysters are morphologically different to those of mussels, with the same adhesive functions forming a more cement-like secretion from the larvae stage onwards so that the ventral valve of the oyster shell adheres to the substrate (Prytherch, 1934). Both insoluble protein (spot 279) and shematrin-7 (spot 290) affect the crystallization of $CaCO_3$ and are known to regulate the calcification of the prismatic layer of pearl oyster, *Pinctada fulcata*, shells (Suzuki et al., 2004; Yano et al., 2006; Suzuki and Nagasawa, 2007) with granular haemocytes in *C. virginica* haemolymph shown to be involved in shell regeneration (Mount et al., 2004). Hence, changes in the concentrations of non-gradient byssal precursor, insoluble protein and shematrin-7 detected in the current study might significantly alter shell properties. Other studies have suggested the capacity of *P. imbricata* to accumulate Pb in its shell make shell properties a good monitor for Pb exposure (MacFarlane et al., 2006). The current study suggests that Zn exposure affects shell property proteins in oysters at cellular levels at low concentrations over short time periods making this suite of proteins an ideal early warning for Zn contamination.

Metal (most prevalently Pb) exposure also affected three proteins with roles in energy metabolism and/or stress response; omega-crystallin, vitellogenin and triosephosphate isomerase. Triosephosphate isomerase (TIM) significantly increased in intensity (2.3-fold). TIM is a glycolytic enzyme essential for efficient energy metabolism (Nagano et al., 2002). It is suggested that increased amounts of TIM in haemocytes of the mosquito, *Anopheles gambiae*, may be linked to an increase in metabolic activity (Paskewitz and Shi, 2005). The concentration of vitellogenin increased by 4.6-fold after oysters were exposed to Pb. Its utility as a molecular biomarker to monitor the effects of estrogenic endocrine disrupting contaminants has been studied extensively (Blaise et al., 1999; Gagné et al., 2001; Matozzo et al., 2008; Gagnaire et al., 2009). The current study shows that vitellogenin-like proteins are also present at differential concentrations after exposure to metal contaminants. Omega crystallin levels decreased by 2-fold in response to Cu exposure. This protein is closely related to aldehyde dehydrogenase (Carosa et al., 2002), a multifunctional enzyme that has been characterized in the gills and mantle of molluscs where its role is often related to protection against physiological stress (Piatigorsky et al., 2000). Omega-crystallin, TIM and vitellogenin all feed into the central pathway for ATP production in energy metabolism. In

C. virginica alterations in standard metabolic rates led to impaired stress tolerance (Lannig et al., 2006). Given the number and levels of changes seen in this study, primarily in response to Pb exposure, energy metabolism appears to be compromised in *S. glomerata* potentially making oysters more susceptible to stress.

The protein analyses in the current study show some similarities to those found in other proteomic studies in which organisms were exposed to contaminants other than metals. Altered concentrations of aldehyde dehydrogenase (omega-crystallin) have been identified by 2DE analysis of *M. edulis* digestive gland peroxisomes after exposure to a range of marine pollutants (Apraiz et al., 2006) whilst vitellogenin levels increase in *S. glomerata* exposed to estrogenic compounds (Andrew et al., 2008). In contrast, some differentially expressed proteins that have often been associated with stress in other species were not identified in the current study. For instance, HSP60 expression has been associated with Cu exposure in *M. edulis* mantle tissue (Sanders et al., 1991) and HSP90 and metallothionein expression in *C. gigas* digestive glands increase after exposure to Cd (Choi et al., 2008). Our failure to identify these common stress response proteins in *S. glomerata* after metal exposure may be due to the types of tissues analysed (haemolymph in the current study versus mantle and digestive tissue in other studies), or that different concentrations of contaminants evoke different protein responses.

In conclusion, the current study has identified some overlap between differentially expressed proteins affected by three different metals, and with other environmental proteomic studies. However, its most striking result is that very distinct sets of proteins were significantly altered after exposure to Cu, Pb or Zn. In the case of Pb and Zn, the specific biological functions these distinct protein sets are associated with indicate that different metals affect different cellular pathways. Overall, the data suggests that proteomics provides a useful framework to develop new biomonitoring tools that can differentiate between different types of environmental contaminants.

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