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Proteomic discovery of biomarkers of metal contamination in Sydney Rock oysters (*Saccostrea glomerata*)

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ABSTRACT

In the current study we examined the effects of metal contamination on the protein complement of Sydney Rock oysters. *Saccostrea glomerata* were exposed for 4 days to three environmentally relevant concentrations (100 μg/l, 50 μg/l and 5 μg/l) of cadmium, copper, lead and zinc. Protein abundances in oyster haemolymph from metal-exposed oysters were compared to those from non-exposed controls using two-dimensional electrophoresis to display differentially expressed proteins. Differentially expressed proteins were subsequently identified using tandem mass spectrometry (LC–MS/MS), to assign their putative biological functions. Unique sets of differentially expressed proteins were affected by each metal, in addition to proteins that were affected by more than one metal. The proteins identified included some that are commonly associated with environmental monitoring, such as HSP 70, and other novel proteins not previously considered as candidates for molecular biomonitoring. The most common biological functions of proteins were associated with stress response, cytoskeletal activity and protein synthesis.

1. Introduction

Metal contamination from anthropogenic sources poses a significant threat to the biological sustainability of coastal waterways around the world (Li et al., 2000). A variety of potentially toxic metals enter coastal waterways via urban and industrial processes. These include copper (Cu) from electrical products and agricultural fertilizers; zinc (Zn) from car tyres and pharmaceuticals; and cadmium (Cd) from household batteries (Birch and Taylor, 1999; Snowdon and Birch, 2004). In Australia, metal contamination in the marine environment poses a particularly significant threat because over 86% of the population lives in heavy urbanised areas within 3 km of coastal rivers, estuaries or embayments (Zann, 1996). Persistent and ongoing metal contamination has led government regulatory authorities to seek effective methods to monitor contamination at both the geochemical and biological levels.

Chemical analyses of sediments provide fine-scale contaminant mapping of waterways (Birch and Taylor, 1999; Scanes and Roach, 1999) which allows historical and current day levels of contaminants to be mapped. However, such geochemical analyses do not give an accurate assessment of the potential effects of metal contamination on the biota that inhabit coastal waterways, nor on the possible impacts on their ecosystems (Gray, 1992).

Ecotoxicological techniques that assess mortality or morbidity among key indicator species provide some information about the potential biological impacts of metal contamination (Sanders et al., 1998; Stark, 1998). However, these types of ecotoxicological tools are generally endpoint assays that identify effects only at acute levels of toxicity (Newton and Bartsch, 2007; Roman et al., 2007). They do not provide information about the cellular and physiological processes affected by metal contamination at sub-lethal concentrations that might affect ecosystem function without resulting in mortality or morbidity.

In contrast, biomarkers can be measured from cellular to ecosystem level. The use of molecular biomarkers has the potential

Abbreviations: Cd/$CdCl_2$, cadmium chloride; Cu/$CuCl_2$, copper chloride; Pb/$PbCl_2$, lead chloride; Zn/$ZnCl_2$, zinc chloride; 2-DE, two-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; HSP, heat shock protein.

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to identify cellular effects of metal contamination at sub-lethal levels. For instance, metallothionein and heat shock protein (HSP) levels have been used in field studies as indicators of sub-lethal Cd and Cu contamination in the crab *Carcinus maenas* (Pedersen and Lundebye, 1996), and as indicators for Cd, Cu and Zn stress in the freshwater mussel *Pyganodon grandis* (Giguère et al., 2003). Changes in the expression of molecular biomarkers reflect an organism's initial response to environmental perturbation. They reveal biochemical or physiological effects on organisms and thus show a depth of biological information on the impact of contaminants (van der Oost et al., 1996).

Despite its increasing adoption in weight-of-evidence approaches to environmental monitoring, the effectiveness of molecular biomonitoring is limited by the restricted suites of available biomarkers. Most of the currently used biomarkers are generic molecules that are thought to have similar potentialities across a broad range of species. These include molecules such as HSPs that act as molecular chaperones during cellular stress responses, and metallothioneins, which are a family of cysteine-rich proteins that interact with a range of physiological and xenobiotic metals. Even though the currently used molecules represent effective biomarkers over a range of species, it is likely that there are other tissue- or species-specific genes and proteins that may be more effective molecular biomonitoring tools. A species relevant to the study area also makes the study ecologically relevant (Wu et al., 2005).

In this study we used two-dimensional protein gel electrophoresis (2-DE) to discover novel protein biomarkers of metal contamination in Sydney Rock oysters (*Saccostrea glomerata*) that may be more sensitive or specific than the currently limited range of molecular biomarkers used in other species. *Saccostrea glomerata* is a ubiquitous and ecologically relevant species in NSW estuaries, and is the focus of a major aquaculture industry. Bivalve molluscs 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). Their ability to bio-accumulate contaminants quickly and depurate them slowly makes them an ideal test species for this study (Brown and McPherson, 1992; Hardiman and Pearson, 1995; Scanes, 1997; Scanes and Roach, 1999).

Proteomic approaches are powerful tools for biomarker discovery in environmental science (Viant et al., 2002; Jonsson et al., 2006). Large numbers of proteins can be analysed simultaneously, potentially providing complex protein expression signatures for individual chemicals and synergistic exposures (Bradley et al., 2002; Rodríguez-Ortega et al., 2003; Apraiz et al., 2006). This allows subtle environmental changes to be detected at low levels and provides quantitative information on the responses of individual proteins and underlying biological activities of these proteins (Nesatyy and Suter, 2007). Such approaches have already been used previously to detect biomarkers for contaminant assessment, in the mussel, *Mytilus edulis* (Apraiz et al., 2006), the oyster, *Crassostrea virginica* (Cruz-Rodríguez and Chu, 2002), and the clam, *Chamaelea gallina* (Rodríguez-Ortega et al., 2003).

In this study we identified proteins in Sydney Rock oyster haemolymph that are differentially expressed in oysters that had been exposed to a range of environmentally relevant concentrations of Cd, Cu, Pb and Zn under controlled laboratory conditions. The data are analysed in an effort to identify new biomarkers that can discriminate between different types of metal contamination over a broad range of doses.

2. Materials and methods

2.1. Oyster acclimation and exposure to metals

Eighteen month to 2 year old Sydney Rock oysters were purchased from Aquaculture Enterprises (Eden, NSW, Australia). Oysters were placed in 12 × 25 l aquaria at the Sydney Institute of Marine Science (Chowder Bay, Sydney, Australia) with each aquarium containing 7 oysters. They were left to acclimate to aquarium conditions for 10 days prior to metal exposures. Complete water changes were performed daily throughout the duration of the experiment with water taken directly from Chowder Bay, and oysters were fed every 4 days with M-1 bivalve food (Aquasonic, Wauchope, NSW, Australia). Water quality parameters (temperature and salinity) were recorded daily. Prior to the start of the exposure trials, water samples were taken from Chowder Bay and the levels of a suite of metals, polychlorinated biphenyls (PCBs), and poly-aromatic hydrocarbons (PAHs) were assessed by the Australian Government National Measurement Institute (Pymble, NSW, Australia).

Following the 10 day acclimation period, oysters were exposed for 4 days to 100 μg/l (three aquaria), 50 μg/l (three aquaria) and 5 μg/l (three aquaria) of $CdCl_2$. The remaining three aquaria were used as controls with no added metal. The experiment was repeated three times using $CuCl_2$, $PbCl_2$ then $ZnCl_2$. Stock solutions of metals were dissolved in seawater before being added to aquaria and water containing metals was changed daily.

2.2. Protein extraction

After exposure to metals, oysters were shucked and 500 μl of haemolymph was harvested from the pericardial cavity using a micropipette. The haemolymph was immediately mixed with 1.3 ml of Tri-reagent LS (Sigma–Aldrich). 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 removed by adding 300 μl of 100% ethanol for 3 min followed by centrifugation at 2,000 × *g* for 5 min (4 °C), after which the DNA pellet was discarded. Finally, proteins were precipitated by adding 3× volumes of ice cold acetone, standing samples at room temperature for 10 min, followed by centrifugation for 10 min at 12,000 × *g* (4 °C). The protein pellets were washed by incubation four times in 1 ml 0.3 M guanidine hydrochloride in 95% ethanol (V:V) for 10 min per wash, followed by centrifugation at 8,000 × *g* for 5 min (4 °C) and removal of the supernatant. The resulting protein pellets were then air dried at room temperature before re-suspension in 50 μl re-hydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS); 50 mM dithiothreitol (DTT)).

2.3. Protein quantification and pooling of samples

The concentrations of proteins in the re-suspended pellets were quantified using Amersham 2-DE Quant Kits according to manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Briefly, 2 μl of each sample was added to each three wells of a 96-well microtiter plate. Ten microlitres of Cu solution, 40 μl of Milli Q water and 100 μl of colour reagent was also added to each well and the plate was left to incubate at room temperature for 20 min. Absorbance was measured at 490 nm on a microplate reader and protein concentrations were interpolated from a standard curve generated with bovine serum albumin. Haemolymph from five randomly selected oysters in each aquarium were pooled to give a total of three replicates per metal concentration (each replicate

representing a single aquarium) plus three replicates of controls. Each pooled haemolymph sample contained 150 μg of protein based on relative protein concentrations of each individual oyster.

2.4. Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis was performed according to the method of Thompson et al. (2011). Briefly, isoelectrofocusing (IEF) was undertaken using immobilized pH linear gradient gel strips (7 cm, pH 4–7; GE Healthcare) on an IPGphor IEF system (GE Healthcare). Gel strips were passively re-hydrated overnight with 150 μg of extracted proteins 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. Gel strips were then reduced (1% DTT, 20 min) and alkylated (2.5% iodoacetamide, 20 min). Second dimension separation was undertaken using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% Tris–HCl polyacrylamide gels (1.5 M Tris–HCl, 10% SDS, 12% acrylamide) in a Mini PROTEAN system (Bio-Rad). Gels were stained with Lava Purple (Fluorotechnics, Sydney, Australia) and visualised using a Typhoon Trio laser scanner (GE Healthcare).

2.5. Protein spot analysis

Progenesis proteomic analysis software (Non Linear Dynamics, Newcastle-upon-Tyne, UK) was used to identify significant changes ($p < 0.05$) in protein spot intensity between treatments. Fluorescence intensities of protein spots on gel maps from each metal at each concentration were compared to intensities of corresponding proteins on gels from controls. Fold differences for each spot that differed in intensity between metal-exposed oysters and controls were calculated from the mean normalized spot volumes of controls compared to the respective metal treatment.

2.6. In-gel digestion of proteins from 2-DE gels

Spots selected for further analysis were picked from gels and digested using trypsin. Each gel plug was washed three times for 10 min with 100 mM ammonium bicarbonate (NH_4HCO_3). Gel plugs were de-stained in 50% acetonitrile (ACN)/50 mM NH_4HCO_3 then dehydrated in 100% ACN for 5 min and air dried. They were then 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 before being washed and dehydrated as described above. Thirty microlitres trypsin solution (12.5 ng/μl in 50 mM NH_4HCO_3, Promega, Sydney, Australia) were added for 30 min at 4 °C, before the gel plugs were incubated overnight at 37 °C. The gel plugs were then washed twice in 50% ACN/2% formic acid for 30 min to extract tryptic peptides. The resulting supernatants (50–60 μl) containing peptides were reduced to 12 μl in a vacuum centrifuge then further centrifuged for 10 min at 14,000 rpm to remove microparticles.

2.7. Nanoflow liquid chromatography–tandem mass spectrometry

Tryptic peptides from excised gel plugs 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 (Andon et al., 2003). Reversed phase columns were packed in-house (approximately 7 cm, 100 μm i.d.) using 100 Å, 5 mM Zorbax C18 resin (Agilent Technologies, CA, USA) in a fused silica capillary using an integrated electrospray tip. A 1.8 kV electrospray voltage was applied via a liquid junction up-stream of the C18 column. A Surveyor autosampler (Thermo, CA, USA) was used to injected samples onto the 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).

2.8. Protein (peptide) identification

Raw MS data files were converted to mzXML format and they were searched through the Global Proteome Machine (GPM) software version 2.1.1 of the X!Tandem algorithm (http://www.thegpm.org/) (Craig and Beavis, 2003, 2004) against a database containing 14,002 peptide sequences from bivalve molluscs (plus common human and trypsin peptide contaminants) downloaded in August 2009 from the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). A search was also performed against a reversed sequence database to evaluate the false discovery rate (FDR). GPM 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 peptides that had $\log(e)^+$ values of <-10 and yielded at least five spectral counts were retained for further analysis. Using these criteria, no non-palindromic reverse database peptide identifications were detected. Identified peptides were assigned a biological function based on functional annotations for the homologous sequences in the NCBI database.

2.9. Statistical analysis

Relationships between proteins expressed in response to each concentration of metal were examined using cluster analysis. Protein spot abundances were $\log(X+1)$ transformed and similarities determined using Bray–Curtis index. All analyses were performed using Primer 6+ statistical software (Plymouth Marine Laboratories, Plymouth, UK).

3. Results

3.1. Water quality data

Average water temperatures throughout each trial were 21.0 °C, 20.0 °C, 22.5 °C and 22.5 °C for Cd-, Cu-, Pb- and Zn-exposures, respectively. There were significant differences in temperature and salinity between the four metal exposure trials. Pb- and Zn-exposures had higher temperatures ($p < 0.05$) than both the Cd- and Cu-exposures. However, the largest difference in temperature was only 2.5 °C (between Pb/Zn and Cu). The Cu-exposure had the lowest average salinity (34 ppt), whilst the highest salinity was in the Pb-exposure (36 ppt). Metal and PCB concentrations in water samples from Chowder Bay were all <2 μg/l, whilst levels of PAHs were <0.1 μg/l.

3.2. Protein expression patterns after metal exposure

An average of 305 distinct protein spots per 2-DE gel was visualised by Progenesis. A total of 129 spots changed significantly ($p < 0.05$) in intensity in response to metal exposure when

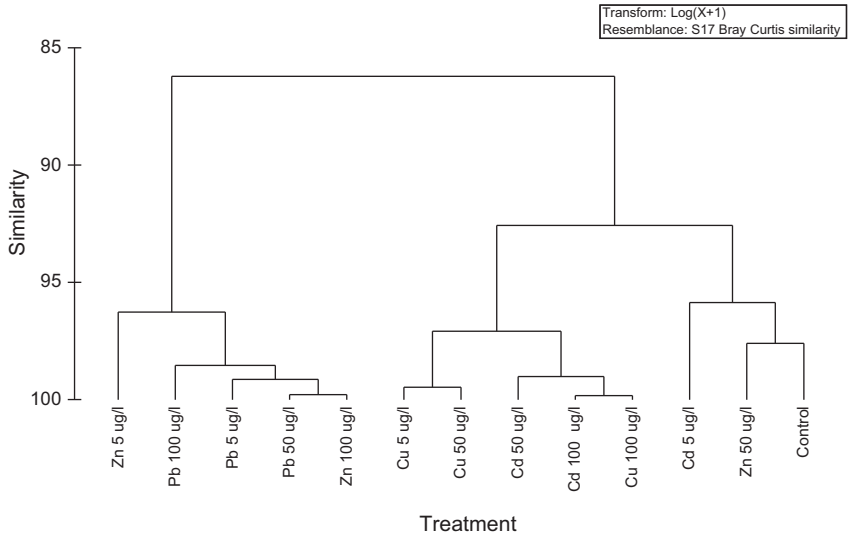

Fig. 1. Dendogram showing similarity of the differential protein expression in response to exposure to Cd, Cu, Pb and Zn at each concentration, relative to controls.

compared to non-exposed controls. Twenty-one of these 129 differential spots differed significantly in intensity between controls and exposure to more than one of the metals. None of the spots changed in intensity in response to all metals when compared to controls. However, 7 spots (spots 2, 4, 36, 68, 270, 271 and 299) changed in response to three of the metals tested and a further 14 spots (spots 9, 18, 25, 27, 35, 42, 55, 64, 67, 74, 108, 109, 276, and 342) varied in response to two metals. All other spots responded in a metal-specific manner.

The total numbers of differential spots were similar for each metal concentration; 45 spots changed in intensity when compared to controls in response to 5 μg/l, 41 spots varied in response to the 50 μg/l exposures, and 43 spots changed in intensity in response to 100 μg/l metal. When metal exposure was analysed, Cd, Cu, Pb and Zn resulted in significant changes in intensities of 34, 24, 38 and 33 spots, respectively, with the number of spots altered in response to Cu being significantly (<0.05) lower than for Pb.

Multivariate cluster analysis (Fig. 1) shows there was similarity in the proteins expressed by Pb and Zn (except for Zn at 50 μg/l). There was also similarity in those proteins expressed by Cd and Cu (except for Cd at 5 μg/l). However, these two groups of proteins were distinctly separate to each other.

3.3. Metal-specific protein expression

Fig. 2 shows that there were dose-dependent changes in the number of protein spots altered by each metal. There was a significant increase in the number of spots altered in response to Cd as concentration increased. An opposite effect was evident in oysters exposed to Cu, which had a greater impact on the number of spots that changed relative to controls at the lowest concentration (5 μg/l) compared to the highest concentration (100 μg/l). After Pb exposure, significantly more spots changed in intensity in response to 50 μg/l compared to 5 μg/l. Zn exposure resulted in more spots responding to 5 μg/l than to 50 μg/l.

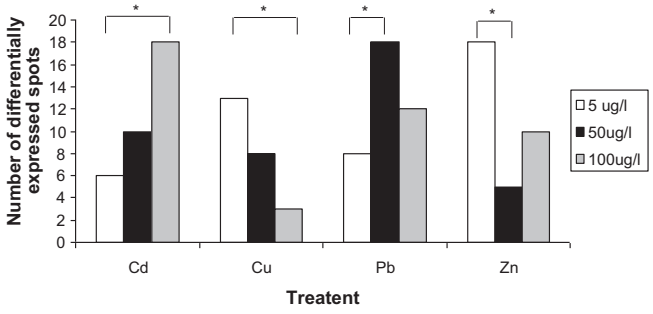

Fig. 2. The total number of proteins spots that were differentially expressed between metal-exposed oysters and controls at each metal concentration. *Indicates statistically significant differences.

Of the 129 differential proteins detected, 89 decreased in intensity after metal exposure whilst 40 increased in intensity. In Pb-exposed oysters, approximately the same number of spots increased or decreased in intensity (17 increased and 22 decreased). Cu and Zn exposures resulted in a far greater proportion of spots that decreased in intensity (80% and 70% decreasing, respectively).

Fig. 3 shows the fold differences in intensity of individual protein spots at each concentration of metal. Spot 299 had the highest fold difference of any spot relative to controls. It decreased in intensity in response to Cu by 46-fold, to Pb by 38-fold and to Zn by 40-fold. Fold differences for all other spots ranged between 0.45 (spot 270, in response to Cu) and 8.25 (spot 349 in response to Zn).

3.4. Protein identification

Putative identifications were obtained for 47 unique differential spots when peptide spectra were compared to sequences in the custom *Bivalvia* sequence database. Details of these putative identifications are shown in Table 1. Among these, 33 spots were found to match a single peptide. The peptide spectra of the remaining spots matched more than one peptide sequence in the database so that changes in the relative intensities of those spots could not be unequivocally assigned to an individual protein. However, some proteins, such as 40S ribosomal protein, appeared in a number of the spots that contained matches to more than one peptide. There was further redundancy in the identifications meaning that the same peptide was sometimes identified in more than one spot. As a result, peptides closely related to 20 unique proteins were identified among the 33 spots that contained a single peptide.

Several of the identified proteins responded to just one metal. Arginine kinase, vitellogenin, triosephosphate isomerase and myosin essential light chain changed in intensity only after exposure to Pb, whilst shematrin-7, insoluble protein and mitochondrial mortalin were altered only after exposure to Zn.

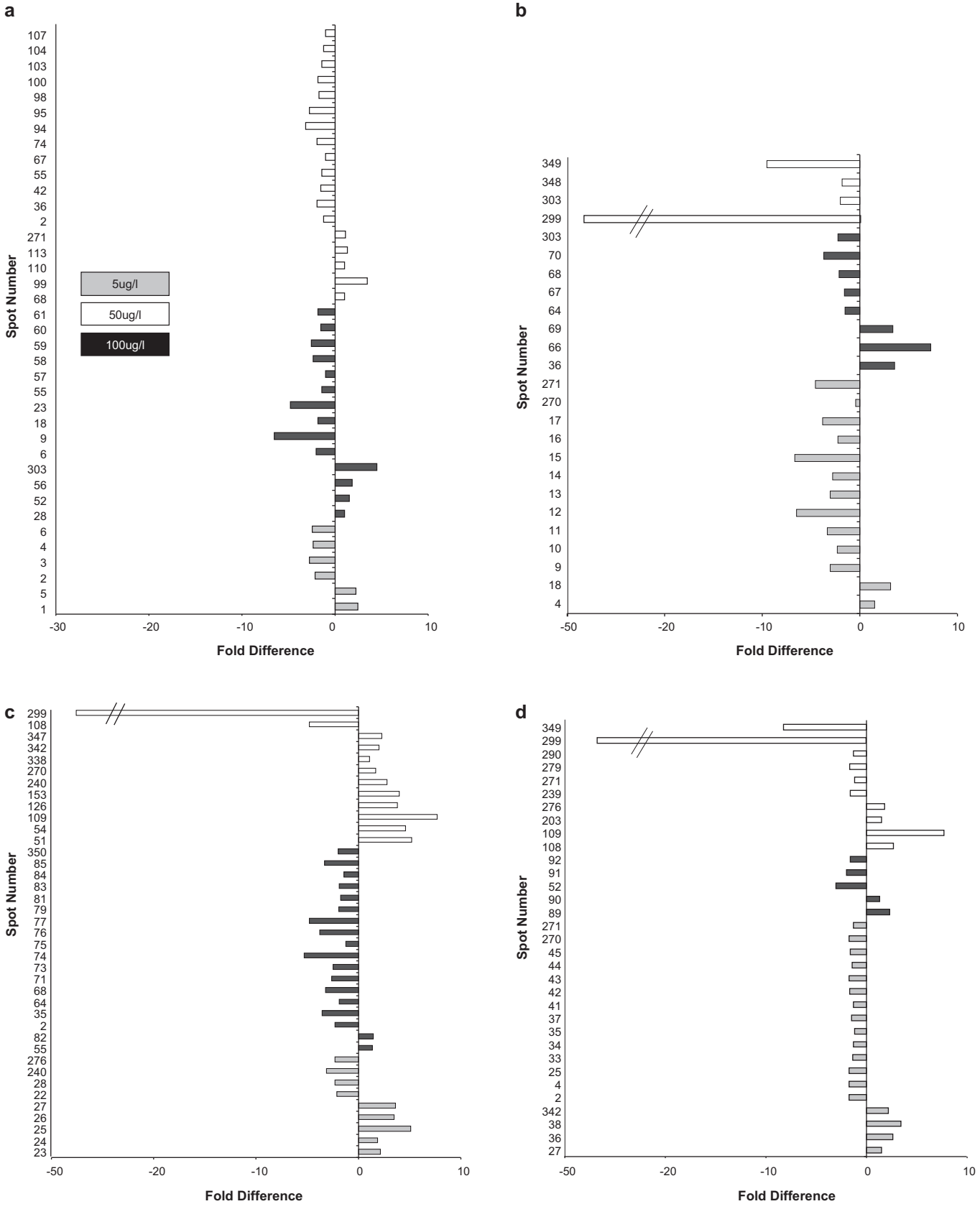

Fig. 3. (a–d) Fold differences relative to non-exposed controls in spots that differed significantly ($p < 0.05$) in intensity between metal-exposed oysters and non-exposed controls: a, Cd; b, Cu; d, Pb; d, Zn.

Table 1
Average normalized spot volumes, putative identifications of proteins in the NCBI bi-valvia database for spots that differed in intensity ($p < 0.05$) between metal-exposed and control oysters.

Metal	Spot #	Conc. (μg/l)	Number of peptides	Log(e)$^+$ value	*T*-test (*p*-value)	Putative identification(s)	Identify of proteins in NCBI database
Cd	2	5	7 7	−73.9 −71.3	0.02	Tropomyosin and ATP synthase	gi|219806594|*Crassostrea gigas*/gi|86156234|*Crassostrea gigas*
	3		5	−55.0	0.001	40S ribosomal protein SA	gi|229891605|*Pinctada fucata*
	5		10	−35.1	0.001	Ferritin	gi|93139010|*Crassostrea ariakensis*
	9	50	5	−33.4	0.01	Vasa	gi|91179150|*Chlamys farreri*
	18		21	−293.2	0.01	HSP70	gi|7688162|*Crassostrea virginica*
	55		7	−60.3	0.001	G protein beta subunit	gi|46391574|*Pinctada fucata*
	56		5 5	−37.2 −33.7	0.03	G protein beta subunit and cytoplasmic HSP 70	gi|46391574|*Pinctada fucata*/ gi|21311271|*Ostrea edulis*
	303		5	−39.2	0.01	Omega crystallin	gi|9957079|*Placopecten magellanicus*
	2	100	7 7	−73.9 −71.3	0.02	Tropomyosin and ATP synthase	gi|219806594|*Crassostrea gigas*/gi|86156234|*Crassostrea gigas*
	36		47 15	−675.3 −167.3	0.05	71 kDa heat shock protein connate and Beta-actin	gi|46359616|*Crassostrea gigas*/ gi|159507454|*Crasssostrea ariakensis*
	55		7	−60.3	0.001	G protein beta subunit	gi|46391574|*Pinctada fucata*
	271		16	−116.3	0.05	Non-gradient byssal precursor	gi|3513512|*Mytilus edulis*
Cu	9	5	5	−33.4	0.001	Vasa	gi|91179150|*Chlamys farreri*
	10		5 5	−31.9 −62.7	0.01	Alanopine dehydrogenase and cytosolic malate	gi|113927399|*Crassostrea gigas*/gi|73656362|*Mytilus californianus*
	11		5	−49.5	0.02	Cytosolic malate	gi|73656362|*Mytilus californianus*
	12		13	−123.2	0.01	Actin	gi|18565104 *Crassostrea gigas*
	14		10 8 8	−91.0 −85.7 −74.4	0.02	Alanopine dehydrogenase, tektin and actin	gi|113927399|*Crassostrea gigas*/ gi|194068377|*Saccostrea kegaki*/ gi|159507454|*Crasssostrea ariakensis*
	15		31	−380.6	0.01	Actin 2/beta-actin	gi|159507454|*Crassostrea ariakensis*
	18		21	−293.2	0.03	HSP70	gi|7688162|*Crassostrea gigas*
	271		16	−116.3	0.01	Non-gradient byssal precursor	gi|3513512|*Mytilus edulis*
	36	50	47 15	−675.3 −167.3	0.01	71 kDa heat shock protein connate and Beta-actin	gi|46359616|*Crassostrea gigas*/ gi|159507454|*Crasssostrea ariakensis*
	303		5	−39.2	0.001	Omega crystallin	gi|9957079|*Placopecten magellanicus*
	299	100	6	−38.4	0.005	Vasa	gi|91179150|*Chlamys farreri*
	303		5	−39.2	0.001	Omega crystallin	gi|9957079|*Placopecten magellanicus*
Pb	22	5	5 17	−39.7 −155.3	0.02	Actin and extracellular SOD	gi|18565104|*Crassostrea gigas*/ gi|229485195 *Saccostrea glomerata*
	23		5 5 5	−31.6 −54.0 −80.1	0.009	NADH dehydrogenase, extracellular SOD and 40S ribosomal protein	gi|225393109|*Pleurobema clava* gi|229485195 *Saccostrea glomerata*/ gi|229891605|*Pinctada fucata*
	24		14	−166.4	0.02	40S ribosomal protein SA	gi|229891605|*Pinctada fucata*
	27		6	−47.6	0.009	Ferritin	gi|32479251|*Crassostrea gigas*

Table 1 (*Continued*)

Metal	Spot #	Conc. (μg/l)	Number of peptides	Log(e)$^+$ value	*T*-test (*p*-value)	Putative identification(s)	Identify of proteins in NCBI database
	28		6		0.02	HSP70	gi|42494889|*Mizuhopecten yessoensis*
	240		17	−46.1	0.001	Myosin essential light chain	gi|40642994|*Crassostrea gigas*
	276		5 6	−30.7 −42.1	0.12	Polyprotein and pre-collagen D	gi|124055243|*Mizuhopecten yessoensis*/ gi|21105303|*Mytilus galloprovincialis*
	2	50	7 7	−73.9 −71.3	0.01	Tropomyosin and ATP synthase	gi|219806594|*Crassostrea gigas*/gi|86156234|*Crassostrea gigas*
	35		83 25	−1103.5 −92.4	0.01	71 kDa heat shock protein connate and Beta-actin	gi|46359616|*Crassostrea gigas*/ gi|159507454|*Crassostrea ariakensis*
	55		7	−60.3	0.04	G protein beta subunit	gi|46391574|*Pinctada fucata*
	75		12 6	−103.3 −54.3	0.03	Tubulin and extracellular SOD	gi|58219310|*Crassostrea gigas*/ gi|229485195|*Saccostrea glomerata*
	76		8	−73.9	0.01	Tropomyosin	gi|219806594|*Crassostrea gigas*
	79		6	−56.0	0.01	Arginine kinase	gi|44885729|*Crassostrea gigas*
	81		5	−36.5	0.001	Cytosolic malate dehydrogenase	gi|73656362|*Mytilus californianus*
	82		6	−51.9	0.005	Extracellular SOD	gi|229485195|*Saccostrea glomerata*
	54	100	5	−39.9	0.02	Vitellogenin	gi|158515777|*Saccostrea glomerata*
	108		10 5	−73.0 −30.9	0.03	Non-gradient byssal precursor and SERCA	gi|3513512|*Mytilus edulis*/ gi|152003987|*Pinctada fucata*
	109		31	−402.2	0.006	Actin	gi|3182893|*Crassostrea gigas*
	126		122	−223.2	0.02	40S ribosomal protein SA	gi|229891605|*Pinctada fucata*
	153		5	−39.9	0.04	Tropomyosin	gi|15419048|*Crassostrea gigas*
	240		17	−46.1	0.001	Myosin essential light chain	gi|40642994|*Crassostrea gigas*
	299		6	−38.4	0.005	Vasa	gi|91179150|*Chlamys farreri*
	338		6 5	−55.2 −41.9	0.04	Beta tubulin and omega crystallin	gi|56603670|*Crassostrea gigas*/gi|9957079|*Placopecten magellanicus*
	342		37 28	−412.1 −56.0	0.005	Tubulin and ATP synthase	gi|194068375|*Saccostrea kegaki*/gi|86156234|*Pinctada fucata*
	347		16	−175.7	0.01	Triosephosphate isomerase	gi|46909461|*Mytilus edulis*
Zn	2	5	7 7	−73.9 −71.3	0.02	Tropomyosin and ATP synthase	gi|219806594|*Crassostrea gigas*/gi|86156234|*Crassostrea gigas*
	27		6	−47.6	0.03	Ferritin	gi|32479251|*Crassostrea gigas*
	33		18	−187.0	0.03	71 kDa heat shock protein connate	gi|46359616|*Crassostrea gigas*
	34		22	−242.0	0.02	71 kDa heat shock protein connate	gi|46359616|*Crassostrea gigas*
	35		83 25	−1103.5 −92.4	0.05	71 kDa heat shock protein connate and Beta-actin	gi|46359616|*Crassostrea gigas*/ gi|159507454|*Crassostrea ariakensis*
	36		47 15	−675.3 −167.3	0.04	71 kDa heat shock protein connate and Beta-actin	gi|46359616|*Crassostrea gigas*/ gi|159507454|*Crassostrea ariakensis*
	44		5	−30.5	0.02	Mitochondrial mortalin	gi|147907866|*Mya arenaria*
	45		6	−38.9	0.005	HSP70	gi|42494889|*Mizuhopecten yessoensis*
	271		16	−116.3	0.001	Non-gradient byssal precursor	gi|3513512|*Mytilus edulis*

Table 1 (*Continued*)

Metal	Spot #	Conc. (μg/l)	Number of peptides	Log(e)⁺ value	*T*-test (*p*-value)	Putative identification(s)	Identify of proteins in NCBI database
	342		37 28	−412.1 −56.0	0.04	Tubulin and ATP synthase	gi|194068375|*Saccostrea kegaki*/ gi|86156234|*Pinctada fucata*
	90	50	8 5 5	−60.7 −50.6 −34.4	0.04	Molluscan prismatic and nacreous layer 88 kDa protein, Cytosolic malate and Vasa	gi|219566992|*Pinctada fucata*/ gi|73656362|*Mytilus californianus*/ gi|194068383|*Saccostrea kegaki*
	91		10	−104.5	0.03	G protein beta subunit	gi|46391574|*Pinctada fucata*
	108	100	10 5	−73.0 −30.9	0.01	Non-gradient byssal precursor and SERCA	gi|3513512|*Mytilus edulis*/ gi|152003987|*Pinctada fucata*
	109		31	−402.2	0.006	Actin	gi|3182893|*Crassostrea gigas*
	203		6 5 5	−39.0 −33.7 −30.0	0.04	Myosin, polyprotein, and non-gradient byssal precursor	gi|13537551|*Mizuhopecten yessoensis*/ gi|3513512|*Mytilus edulis*
	239		10	−38.4	0.02	Non-gradient byssal precursor	gi|3513512|*Mytilus edulis*
	271		16	−116.3	0.01	Non-gradient byssal precursor	gi|3513512|*Mytilus edulis*
	276		5 6	−30.7 −42.1	0.04	Polyprotein and pre-collagen D	gi|124055243|*Mizuhopecten yessoensis*/ gi|21105303|*Mytilus galloprovincialis*
	279		28	−219.2	0.02	Insoluble protein	gi|2204081|*Pinctada fucata*
	290		7	−48.9	0.03	Shematrin-7	gi|93102311|*Pinctada fucata*
	299		6	−38.4	0.004	Vasa	gi|91179150|*Chlamys farreri*

3.5. Biological functionalities

The differentially expressed proteins from spots that contained a single identification were assigned to one of 8 biological functional categories (Fig. 3) based on their probable functional annotations in the NCBI database. Proteins associated with stress responses (heat shock proteins and omega-crystallin) comprised the largest group (21%) followed by protein synthesis (ribosomal proteins and vasa, 17%), cytoskeletal activities (actin, myosin and tropomyosin, 16%), proteins associated with shell properties (insoluble protein, non-gradient byssal precursor and shematrin-7, 14%) and proteins involved in metabolism (arginine kinase, cytosolic malate dehydrogenase and trisosephosphate isomerase, 14%). Other functional categories affected by metal exposures were oxidative stress (2%), signal transduction (14%) and molecular transport (8%) (Fig. 4).

Table 2 shows the associations of each metal exposure with biological function. Proteins associated with protein synthesis were affected by all four metals, with vasa being the most consistently affected protein over a wide range of metals and doses. Vasa (spot 299) also had the largest fold differences of all the differentially expressed proteins (Fig. 3). Stress response proteins were also affected by all four metals, primarily at the lowest two concentrations (5 and 50 μg/l). HSP 70 was the most consistently affected stress protein, predominantly decreasing in intensity among metal exposed oysters compared to controls. Cytoskeletal proteins were primarily affected by Cu and Pb. These proteins decreased in intensity at low metal concentrations and increased in intensity at the highest concentration (100 μg/l). Actin was affected by both Cu and Pb, but only Pb altered the concentrations of myosin and tropomyosin proteins. Proteins associated with shell properties were affected predominantly by exposure to Zn, decreasing in intensity when compared to controls.

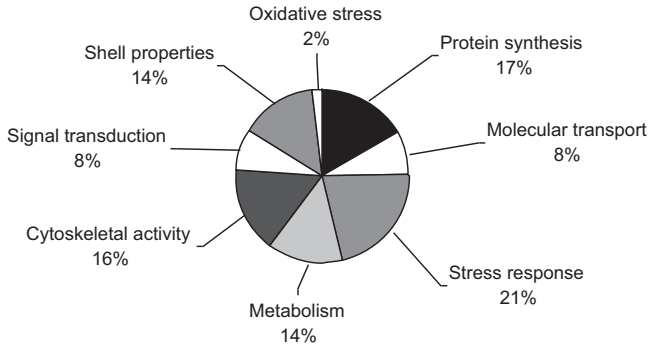

Fig. 4. Biological functions associated with the differential proteins identified by MS. $n = 33$.

4. Discussion

The present study has shown that exposing oysters to different concentrations of a range of metals has substantial impacts on the proteomes of haemolymph. 129 protein spots in *S. glomerata* haemolymph changed significantly in intensity in response to Cd, Cu, Pb and Zn over three concentrations of these contaminants that are known to occur in NSW estuaries (Birch, 2000).

The dose-dependent relationship of proteins affected by Cd suggests that increasing dose rates of Cd have increasing effects on *S. glomerata*. This agrees with a study by Soetaert et al. (2007) who found a dose-dependent increase in the effects of Cd on *Daphnia magna* gene expression. Exposure to Cu in the current study showed the opposite pattern to that of Cd. The number of proteins affected by Cu decreased as the dose rate increased. The effects of Cu have previously been shown to be more toxic at lower concentrations than Cd in *Mytilus galloprovincialis* (Gómez-Mendikute and Cajaraville, 2003).

Table 2
Up (↑) or down (↓) regulation of identified proteins relative to non-exposed controls. Proteins are grouped by biological function.

Biological function	Protein	Cd			Cu			Pb			Zn		
	Concentration	5	50	100	5	50	100	5	50	100	5	50	100
Stress response	HSP70		↓		↑			↓			↓		
	HSP71										↓		
	Mitochondrial mortalin										↓		
Protein synthesis	40S ribosomal protein SA	↓						↑		↑			
	Vasa		↓		↓		↓			↓			↓
Cytoskeletal activity	Actin				↓					↑			↓
	Actin 2/beta-actin				↓								
	Myosin essential light chain							↓		↑			
	Tropomyosin								↓	↑			
Shell properties	Non-gradient byssal precursor			↑	↓						↓		↓
	Insoluble protein												↓
	Shematrin-7												↓
Metabolism	Arginine kinase								↓				
	Malate dehydrogenase				↓				↓				
	Omega crystalline		↑			↓	↓						
	Triosephosphate isomerase									↑			
Signal transduction	G protein beta subunit		↓	↓					↑			↓	
Molecular transport	Ferritin	↑						↑			↑		
	Vitellogenin									↑			
Oxidative stress	Extracellular SOD								↑				

Of the four metals analysed in this study, Pb induced the highest number of protein changes overall. Pb had the greatest effect at 50 μg/l and thus did not show a linear dose–response relationship to the concentrations of metals used in this study. Likewise, the number of protein spots affected by Zn exposure did not fit a linear dose–response.

The cluster analysis suggested that the proteome profiles of differentially expressed proteins of exposed oysters compared to controls were related to metal exposure. Overall, the concentrations of each specific metal exposure were similar to each other. Other proteomic studies have also found similarities in protein expression to be more prevalent in exposure type than concentration of exposure (Apraiz et al., 2006; Gomiero et al., 2006). The similarity of Pb/Zn and Cd/Cu and the apparent separation between these groupings could be attributed to the difference in temperature observed during experimentation. Temperatures during Pb and Zn exposure trials were on average 1.5 °C higher than those during Cd exposures and 2.5 °C higher than temperatures in Cu exposures. Alternatively, the clustering may indicate that Pb and Zn have similar cellular effects, as might Cd and Cu.

However, this explanation is not entirely bourne out by the mass spectrometric identification of differentially expressed proteins which confirmed that a range of biological processes were affected by metals. Some functional pathways were affected by a range of metals, whilst others were metal specific. Proteins associated with cytoskeletal activity (actin, actin-2, tropomyosin and myosin) were affected by exposure to Cu, and Pb. In non-muscle cells, actin is involved in phagocytosis, organelle movement and endo/exo-cytosis (Wang et al., 2008) whilst other studies have found alterations of actin after exposure to Cu in *M. galloprovincialis* (Fagotti et al., 1996; Gómez-Mendikute and Cajaraville, 2003). Our study is the first to highlight the effects of Pb exposure on cytoskeletal activities. Among other cytoskeletal proteins affected, tropomyosin regulates motility and contractile functions in cells (Bailey, 1957) whilst myosin is associated with cell motility and organelle transport (Sellers, 2000). The disturbance of these three key cytoskeletal proteins by Cu or Pb suggest that these metals are likely to affect the motility and plasticity of *S. glomerata* haemocytes with ramifications for cellular processes such as wound healing and phagocytosis.

Exposure to Cd, Cu or Pb all affected pathways involved in energy metabolism. Cytosolic malate dehydrogenase was affected by more than one metal, whilst changes in arginine kinase and triosephosphate isomerase (TIM) abundance were unique to Pb exposure. Malate dehydrogenase is an enzyme in the krebs cycle where it catalyses the conversion malate into oxaloacetate and in the oyster, *C. virginica,* it is the most oxidized intermediate (Ulrich and Marsh, 2006). Changes in malate dehydrogenase have been linked to the high variability of the intertidal environments in the oyster, *C. virginica* (Ulrich and Marsh, 2006). It has been shown to assist in anaerobic metabolism by reoxidizing NADH in bivalves (Zwann et al., 1981; Zubkoff and Ho, 1982), and correlates significantly with respiration in the mussel, *Mytilus californianus* (Dahlhoff et al., 2002). TIM is a glycolytic enzyme essential for energy maintenance in the heart of the mollusc, *Tapes watlingo* (Jamieson and de Rome, 1979). Such glycolytic enzymes, have been shown to be affected by seasonal changes in *C. virginica* (Greenway and Storey, 1999). Arginine kinase facilitates the phosphorylation of arginine to ATP and so may also be related to changes of stress tolerance in oysters (Seals and Grossman, 1988). In the oyster, *C. virginica*, changes in metabolic rates have resulted in a lowered stress tolerance (Lannig et al., 2006). Thus, the significant changes in these enzymes after metal exposure have the potential make *S. glomerata* susceptible to stress.

Proteins associated with shell properties were impacted primarily after exposure to Zn. These included insoluble protein, shematrin-7 and non-gradient byssal precursor. Whilst non-gradient byssal precursor was differentially expressed in response to all metals, both insoluble protein and shematrin-7 were only affected after oysters were exposed to Zn. Non-gradient byssal precursor proteins have adhesive properties that may be affected by Cu exposure in the mussel, *P. viridis* (Nicholson and Lam, 2005). Shematrin-7 and insoluble protein are both involved in the calcification and regeneration of oyster shells (Mount et al., 2004; Suzuki and Nagasawa, 2007). The significant decrease in the abundance of these proteins suggests that Zn exposure has the potential to affect shell adhesion, calcification and regeneration in *S. glomerata*. This differential expression in more than one concentration of Zn used in this study also suggests these proteins may represent robust biomarkers for Zn contamination.

Only two protein functional categories were affected by exposure to all four metals, stress responses and protein synthesis. Vasa, which has been shown to have a role in protein synthesis with RNA metabolism in *C. galloprovincialis* (Venier et al., 2006), was the most consistently affected protein identified in this study. It was affected by all metals over a range of doses, and had the highest fold differences between controls and metal exposures. This suggests that vasa might represent an effective biomarker of metal contamination.

Omega crystallin changed in response to Cd and Cu exposure. It has been linked to physiological stress in molluscs (Piatigorsky et al., 2000) and changes in omega crystallin has been linked to a reduction of temperature stress tolerance in *C. virginica* (Lannig et al., 2006). Heat shock proteins, predominantly HSP 70-like molecules, were affected by all four metals. The induction of the HSP family has been studied extensively after exposure to many contaminants in bivalves including; *C. virginica* after PAH exposure (Cruz-Rodríguez and Chu, 2002), *C. gigas* after exposure to hydrocarbons (Boutet et al., 2004), and *M. edulis* after exposure to tributlytin (Steinert and Pickwell, 1993). The increase in heat shock protein expression has also been specifically linked to metal exposure in *C. gigas* haemolymph after Cd exposure (Choi et al., 2008) and *M. edulis* (Sanders et al., 1991) linked to Cu exposure. An increase in the abundance of these stress response proteins are thought to aid the recovery of metabolic pathways (Rothman, 1989). However, in the current study, HSPs mainly decreased in abundance at low concentrations of metal exposure. This suggests that the abundance of HSPs might be highly dose dependent. Regardless, our identifications of HSPs associated with exposure to all four metals is consistent with the literature: these proteins (particularly HSP 70) have been used extensively in the past as molecular biomarkers of environmental stress.

The significant changes in abundance of both HSP 70 and vasa in response to all four metals (and the high fold differences of vasa) would suggest both may be suitable candidate biomarkers for non-specific metal contamination. In contrast, changes in the abundance of other proteins were highly specific to individual metals. For instance, changes in intensities of TIM, arginine kinase, myosin essential light chain and vitellogenin were unique to Pb exposure. Vitellogenin is involved in yolk deposition regulated by estrogen receptors. This pathway can be affected by contaminants such as estradiol, which disrupts the endocrine pathway (Gagné et al., 2001). Hence, the merits of using vitellogenin as a biomarker for endocrine disruption in molluscs, including *S. glomerata*, have been studied extensively (Riffeser and Hock, 2002; Andrew et al., 2008; Gagnaire et al., 2009).

Even though the present study has identified a number of traditional (HSP 70) and potentially novel (vasa, TIM, arginine kinase, shematrin-7, and insoluble protein) molecular biomarkers of metal contamination in oysters, the dataset highlights some of the pitfalls of this type of proteomics analysis. 2-DE is a low throughput technique, which limits biological replication. This factor, along with the difficulties inherent in identifying proteins in species without fully sequenced genomes, means that statistically significant differences in protein abundance are difficult to obtain by 2-DE. This also may explain why there was a lack of identified proteins commonly found in other biomarker studies, such as metallothionein (Butler and Roesijadi, 2000; Schiedek et al., 2006; Faria et al., 2009), or proteins associated with oxidative stress (Rodríguez-Ariza et al., 2003; Valavanidis et al., 2006; Duarte et al., 2011). As a result, the data presented here are indicative of new candidate molecular biomarkers in *S. glomerata,* and provide information on the physiological systems that are affected by metal contamination. However, they require additional, high throughput analyses to validate their utility in environmental monitoring.

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