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Proteomic analysis of Sydney Rock oysters (*Saccostrea glomerata*) exposed to metal contamination in the field

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ABSTRACT

This study used proteomics to assess the impacts of metal contamination in the field on Sydney Rock oysters. Oysters were transplanted into Lake Macquarie, NSW, for two weeks in both 2009 and 2010. Two-dimensional electrophoresis identified changes in protein expression profiles of oyster haemolymph between control and metal contaminated sites. There were unique protein expression profiles for each field trial. Principal components analysis attributed these differences in oyster proteomes to the different combinations and concentrations of metals and other environmental variables present during the three field trials. Identification of differentially expressed proteins showed that proteins associated with cytoskeletal activity and stress responses were the most commonly affected biological functions in the Sydney Rock oyster. Overall, the data show that proteomics combined with multivariate analysis has the potential to link the effects of contaminants with biological consequences.

1. Introduction

Estuaries worldwide are under pressure from anthropogenic impacts due primarily to high levels of urbanisation and industrialisation (Birch, 2000; Abrahim and Parker, 2002; Connor and Thomas, 2003). The introduction of waste products into rivers and estuaries has led to significant increases in chemical contamination, including metals, that may have deleterious effects on native biota.

There is a range of chemical or ecotoxicological techniques used to assess the impacts of metal contamination (Wilson and Hyne, 1997; Peter et al., 1998; Birch and Taylor, 1999; Taylor et al., 2004). However, there is still a need for new methods to monitor contaminants in ways that are biologically relevant to the animals and plants that live in coastal waterways as many of the aforementioned techniques either do not measure biological effect or look at endpoint concentrations of metals. Molecular biomarkers can provide information on the cause and effect paradigm of contamination, linking contaminants directly with the biology of the systems that they affect (Depledge and Fossi, 1994). Among the available molecular biomonitoring techniques, proteomics provides a good method to gauge the effects of contamination by identifying and quantifying hundreds of potential biomarkers (individual proteins) simultaneously (Larsen et al., 2006). As such, proteomics has the potential to detect biological effects at extremely low levels of contamination over short time periods, providing an early warning of environmental damage (Rodríguez-Ortega et al., 2003; Apraiz et al., 2006).

Proteomics has been used extensively to analyse the effects of contaminants in laboratory-based exposures (Rodríguez-Ortega et al., 2003; Manduzio et al., 2005; Thompson et al., 2011, 2012). These studies have shown that analysing the entire proteome of an organism after contaminant exposure can uncover numerous potential molecular biomarkers. However, laboratory-based studies usually test individual contaminants in isolation. They do not necessarily reflect conditions in the field where a range of environmental variables may affect the proteomes of native biota. Unfortunately, there are relatively few studies that assess the impacts of contamination on the proteomes of organisms in the field.

Abbreviations: 2-DE, 2-dimensional electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; DTT, dithiothreitol; IAA, iodoacetamide; IEF, isoelectric focussing; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HSP, heat shock protein.

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In this study, the proteomes of Sydney Rock oysters (*Saccostrea glomerata)* transplanted into contaminated sites in Lake Macquarie, NSW, are compared to those of oysters held in relatively non-impacted, control areas (see Section 2.1). We hypothesize that the different levels of metals experienced by oysters between the two treatments will result in different oyster proteomes.

Bivalve molluscs, such as oysters, are good indicators of estuarine pollution. They are filter feeders that readily bio-accumulate contaminants and so have been used in many environmental monitoring studies (Scanes, 1997; Gillikin et al., 2005; Marie et al., 2006; Valdez Domingos et al., 2007). Moreover, Sydney Rock oysters are ubiquitous and ecological relevant in NSW estuaries.

2. Materials and methods

2.1. Site description

Lake Macquarie is an estuarine lake 100 km north of Sydney, NSW, that has been used as a model for metal contamination on Australia's eastern seaboard (Batley, 1987; Paterson et al., 2003; Burt et al., 2007). The lake has an average depth of 6.7 m and is part of a catchment spanning 640 km^2 (Batley, 1987). Industrial development started on the shores of Lake Macquarie in the 1890s. It included mining, a zinc smelter and a coal-fired power station. The lake still remains partly surrounded by bushland although some urbanisation has occurred, mostly on its northern shores (Roach, 2005). The increased levels of metals in the north of the lake have been attributed to the now decommissioned smelter (Roach, 2005). As a result, there is a contamination gradient of Cd, Pb, Zn, Hg and Ag in the lake's sediment, which decreases from north to south. In contrast, Cu is prevalent throughout the lake (Roy and Crawford, 1984; Roach, 2005). Ni, Co, Fe are also found in the lake, but in low concentrations (Roy and Crawford, 1984). The nature of the coastal lagoon's narrow oceanic opening results in low tidal ranges (0.1–0.2 m) and low tidal flushing (Burt et al., 2007). Other variables such as temperature, salinity and pH remain fairly uniform lakewide. Studies addressing the impact of metals on biota suggest that levels of Ag, Cd, Hg, Pb, Se and Zn in the north of the lake have the potential to impact on biota (Barwick and Maher, 2003; Roach, 2005).

2.2. Field sites on Lake Macquarie

Three field trials (FT) were conducted in March and April 2009, and March 2010. These are hereafter designated FT1, FT2 and FT3 respectively. For each trial, twelve weighted baskets each containing 7 Sydney Rock oysters, aged 18 month to 2 years, and from the same broadstock, (purchased from Aquaculture Enterprises Eden, NSW) were transplanted into an area of Lake Macquarie that is known to contain low metal concentrations (Fig. 1). Oysters were left for 10 days at this 'acclimation site'. Three baskets each were then relocated per site to two sites in contaminated areas and two sites in areas with low levels of contaminants, deemed 'control' areas (Table 1 and Fig. 1). The three baskets at each site were placed at least 10 m apart and oysters were held at each site for four days. They were then shucked on-site and 500 μl of haemolymph was harvested from the pericardial cavity of each oyster using a micropipette. Each 500 μl sample of haemolymph was mixed with 1.3 ml of Tri-reagent LS (Sigma Aldrich) and immediately frozen on dry ice, transported to the laboratory and then frozen at −80 °C until use.

2.3. Water quality and sediment analysis

Three surficial sediment samples and six water samples were taken from each treatment area on each sampling day. Sediment samples were collected using plastic tubing pushed approximately 10 cm into the sediment. Water samples for the analysis of metal concentrations in suspended particulate matter (SPM) were taken using 2.5 l bottles that were filled completely. A further set of water samples were taken for dissolved metal analysis. For these, 40 ml water was filtered (0.45 μm) before being mixed with 200 μl, 99% nitric acid. All samples were transported in the dark on ice. Temperature, salinity and pH readings were also recorded at each site using a YSI63 water quality probe (YSI Inc., OH, USA).

Sediment samples were wet sieved through a 63 μm nylon mesh to separate the coarse and fine sediment fractions. Fine fractions were then oven dried at 60 °C for 48 h and weighed. To digest each sample, 0.5 g of fine fraction sediment was mixed with 10 ml de-ionised water and four ml aqua regia (1:2.5 nitric acid: hydrochloric acid). Samples were then placed in a heating block at 130 °C for two hours or until

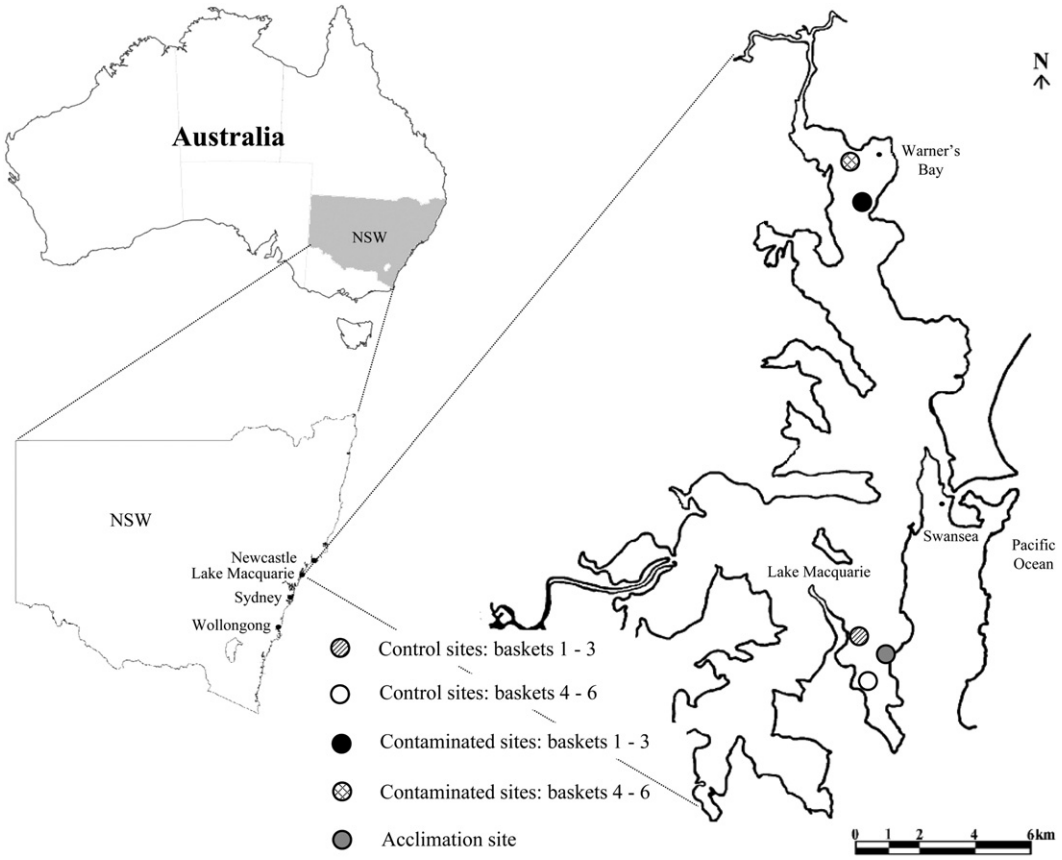

Fig. 1. Lake Macquarie, NSW, showing control and metal contaminated areas and the two sites within each of these areas. The map also shows the site where oysters were acclimated prior to translocation.

Table 1
Longitude and latitudes for each of the control sites and metal contaminated sites in the three field trials.

Treatment area	Longitude (east)	Latitude (south)
Acclimation site	151E 36′ 10.69″	−33S 8′ 34.10″
Control site 1, basket 1	151E 35′ 28.32″	−33S 7′ 54.87″
Control site 1, basket 2	151E 35′ 27.54″	−33S 8′ 1.08″
Control site 1, basket 3	151E 35′ 24.92″	−33S 8′ 4.6″
Control site 2, basket 1	151E 35′ 27.85″	−33S 8′ 27.73″
Control site 2, basket 2	151E 35′ 26.15″	−33S 8′ 39.24″
Control site 2, basket 3	151E 35′ 23.68″	−33S 8′ 43.64″
Contaminated site 1, basket 1	151E 38′ 5.2″	−32S 59′ 24.26″
Contaminated site 1, basket 2	151E 38′ 7.6″	−32S 59′ 19.40″
Contaminated site 1, basket 3	151E 38′ 12.08″	−32S 59′ 17.65″
Contaminated site 2, basket 1	151E 38′ 39.58″	−32S 58′ 40.98″
Contaminated site 2, basket 2	151E 38′ 39.08″	−32S 58′ 38.71″
Contaminated site 2, basket 3	151E 38′ 38.50″	−32S 58′ 35.27″

most of the liquid had evaporated. Once cool, de-ionised water was added up to 30 ml and samples were shaken and left overnight for the sediment to settle. The liquid phase was then nebulised into an 8000 °C plasma and analysed using an inductively coupled plasma-optical emission spectrometer (ICP-OES, Varian/Agilent 720, CA, USA).

Samples for the analysis of SPM were filtered through a manifold onto pre-weighed 0.45 μm cellulose nitrate membranes. Membranes were oven dried overnight at 60 °C and re-weighed. The membranes were then placed into separate tubes, aqua regia solution was added and samples were placed on a heating block as above. De-ionised water was added up to 15 ml and samples were treated as above. All data was then analysed against AGAL 10 industry standards. Several samples were analysed multiple times in addition to blank samples to ensure quality control.

The dissolved metal concentrations in water samples, pre-treated with nitric acid, were determined using a high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS, Finnigan Element 2, Thermo Scientific, USA). These analyses were performed by the National Measurement Institute (Pymble, NSW).

2.4. Protein extraction, quantification and 2-dimensional electrophoresis (2-DE)

The extraction of proteins from haemolymph, quantification, pooling of samples and 2-DE analysis was performed using methods from Thompson et al. (2011). Briefly, RNA was removed from 500 μl haemolymph by adding 100 μl of bromochloropropane for 15 min, centrifugation for 15 min and removal of the colourless aqueous phase. DNA was precipitated by the addition of 300 μl of 100% ethanol for 3 min followed by centrifugation. Proteins in the supernatant were then precipitated by mixing with 3× volumes of ice cold acetone for 10 min, followed by centrifugation for 10 min and removal of the supernatant. Protein pellets were washed four times with 1 ml 0.3 M guanidine hydrochloride in 95% (V:V) ethanol and once in 1 ml 95% ethanol. Finally, protein pellets were re-suspended in 50 μl re-hydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; CHAPS, 50 mM DT dithiothreitol; DTT).

The concentration of proteins in each sample were measured in triplicate using Amersham 2-DE Quant Kits according to manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Two μl of each sample were added per well to 96-well microtiter plates, followed by 10 μl of copper solution, 40 μl of Milli Q water and 100 μl of colour reagent. Plates were then left to incubate at room temperature for 20 min. Absorbance was measured at 490 nm on a microplate reader and protein concentrations were interpolated against a standard curve generated with bovine serum albumin. Five randomly selected haemolymph samples from oysters in each basket were pooled to give a total of three replicates per site (6 replicates per treatment area). Each pooled replicate contained 150 μg of protein.

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 before IEF was performed at 100 V for 2 h, 500 V for 20 min, a gradient up to 5000 V for 2 h then 5000 V for 2 h. 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) so that proteins could be visualised using a Typhoon Trio laser scanner (GE Healthcare). Gels from each trial and each site were run separately.

Progenesis proteomic analysis software (Non Linear Dynamics, Newcastle-upon-Tyne, UK) was used to identify significant changes ($p < 0.05$) in the intensity of protein spots between the haemolymph proteomes of oysters from control and contaminated areas. Normalised fluorescence intensities of protein spots (pixel intensity of spot minus the background pixel intensity of the gel) on gel maps from each control area were compared to intensities of corresponding proteins on gels from contaminated areas using the in-built ANOVA algorithm in Progenesis. Fold differences for each spot that differed significantly in intensity between areas were calculated from mean normalized spot volumes.

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

Differentially expressed protein spots were picked from gels and digested using trypsin. Each gel plug was washed three times for ten minutes with 100 mM ammonium bicarbonate (NH_4HCO_3) then de-stained in 50% acetonitrile (ACN)/50 mM NH_4HCO_3 Gel plugs were then dehydrated in 100% ACN for 5 min, the ACN was removed and gel plugs were 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 above. Thirty microlitres trypsin solution (12.5 ng/μl in 50 mM NH_4HCO_3, Promega, Sydney, Australia) was added to each gel plug for 30 min at 4 °C, before they were incubated overnight at 37 °C. Any remaining solution was transferred into fresh tubes before 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 centrifuged for 10 min at 14,000 rpm to remove microparticles.

2.6. Nanoflow liquid chromatography–tandem mass spectrometry

Tryptic peptides were analyzed 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 (7 cm, 100 μm i.d.) were packed in-house 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) injected samples onto the column followed by an initial wash step with buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) (10 min at 1 μl min^{-1}). Peptides were then 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).

2.7. Protein (peptide) identification

Raw MS data files were converted to mzXML format and searched through the Global Proteome Machine (GPM) software version 2.1.1 of the X!Tandem algorithm, (www.thegpm.org) (Craig and Beavis, 2003, 2004) against a database containing 14,002 peptide sequences from bivalve molluscs, downloaded in August 2009 from the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The database also included peptide sequences from common human and trypsin peptide contaminants. 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 four spectral counts were retained for further analysis. Using these criteria, no non-palindromic reverse database peptide identifications were detected and therefore had an FDR of zero. Identified peptides were assigned a biological function based on functional annotations for the homologous sequences in the NCBI database.

2.8. Statistical analysis

Two-way ANOVAs (with time and location as fixed factors) were used to compare differences in metal concentrations in sediment, SPM and the dissolved fractions between control and contaminated sites in each field trial. Cochrane's tests were used to assess normality (data was log transformed where appropriate) and SNK tests were used for post hoc analysis (GMAV, Institute of Marine Ecology, University of Sydney).

Principal Components Analysis (PCA) was used to show spatial and temporal separation of the metal concentrations in the sediment, SPM and dissolved phase over the three field trials. Contaminant data were normalised prior to analysis. Data for Hg and Ag were omitted from the dissolved phase analysis as concentrations were below detectable limits during all trials.

PCA also examined spatial and temporal separation of the biological data over the three field trials. Protein spot abundances were square-root transformed and inter-sample similarities were determined using Euclidean Distance before analyzing with PCA. Finally, relationships between protein spot profiles and metal contaminants were examined using PCA. Transformed biological data from each field trial was analysed with sediment, SPM, dissolved metals and other environmental variables (temperature, salinity and pH) separately by overlaying the biological data with the respective environmental data. The significance level of all

inferential analyses was set at 0.05. All multivariate analyses were performed using Primer 6+ statistical software (Plymouth Marine Laboratories, Plymouth, UK).

3. Results

3.1. Water quality analysis

The average water temperatures during field trials were 22.5 ± 0.6 °C, 21.4 ± 0.1 °C and 25.3 ± 0.2 °C for FT1, FT2 and FT3 respectively. Average salinities were 34.1 ± 0.1 ppt for FT1, 34.3 ± 0.2 ppt for FT2 and 35.9 ± 0.1 ppt for FT3, and pH was 6.2 ± 0.1, 6.3 ± 0.2 and 6.3 ± 0.1 for each trial. The biggest temperature difference between the control and contaminated sites was 1.8 °C during FT3. Salinity differed between areas by a maximum of 1.3 ppt during FT1. The biggest difference in pH between control (pH 6.3) and contaminated sites (pH 6.1) was during FT1. There were no significant differences in temperature, salinity or pH, between control and contaminated sites in any field trial, or between field trials.

3.2. Differential protein spots

Progenesis software identified an average of 514 spots per oyster proteome. Fifty spots differed significantly in intensity between oysters from control versus contaminated areas. The isoelectric points and molecular weights of these differential spots were randomly distributed in the range pH 4 to 7 and 20–200 kDa (Fig. 2). For each field trial, a unique set of protein spots varied in intensity between control sites and contaminated sites. However, some differential spots were common to more than one field trial (Fig. 3). FT2 had the highest number of protein spots that differed in intensity between control and contaminated areas (24 spots, of which 20 were unique to FT2). Eleven differential spots were found in FT1. Six of these spots were unique to FT1, three differed in intensity in both FT1 and FT2, and a further two differed in intensity during FT1 and FT3. Fifteen differential spots were seen in FT3. Twelve of these were unique to FT3 with the remaining 3 spots overlapping with either FT1 and FT2. There were no spots that varied in intensity between control and contaminated areas in all three field trials.

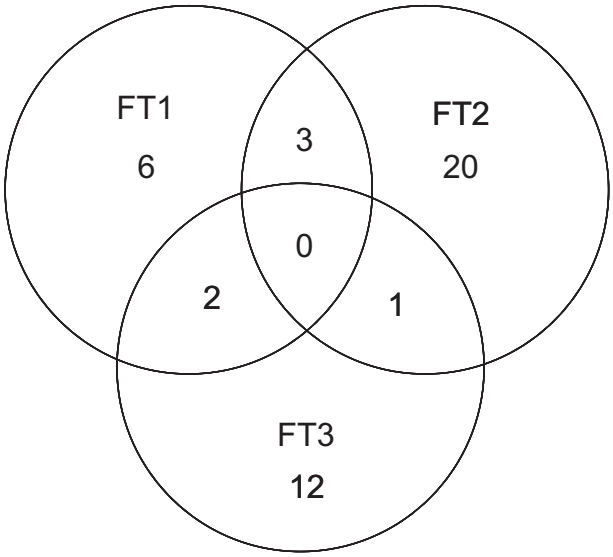

Fig. 3. Venn diagram showing the number of differential protein spots (control compared to contaminated sites) identified in each field trial and the number of differential spots common to more that one field trial.

3.3. Fold differences in spot intensity between control and contaminated treatment areas

Table 2 shows the fold differences in intensity of protein spots that differed significantly between contaminated and control areas in the proteins that were able to be putatively identified. In FT1, fold differences ranged from −1.5 to 2.7. The fold changes in FT2 were between −2.2 and 1.6. In FT3, fold differences ranged from −1.3 to 2.8.

3.4. Putative protein identifications

Of the 44 different protein spots that differed in intensity between control and contaminated sites, a total of 20 spots were able to be identified (6 additional spots were overlaps between

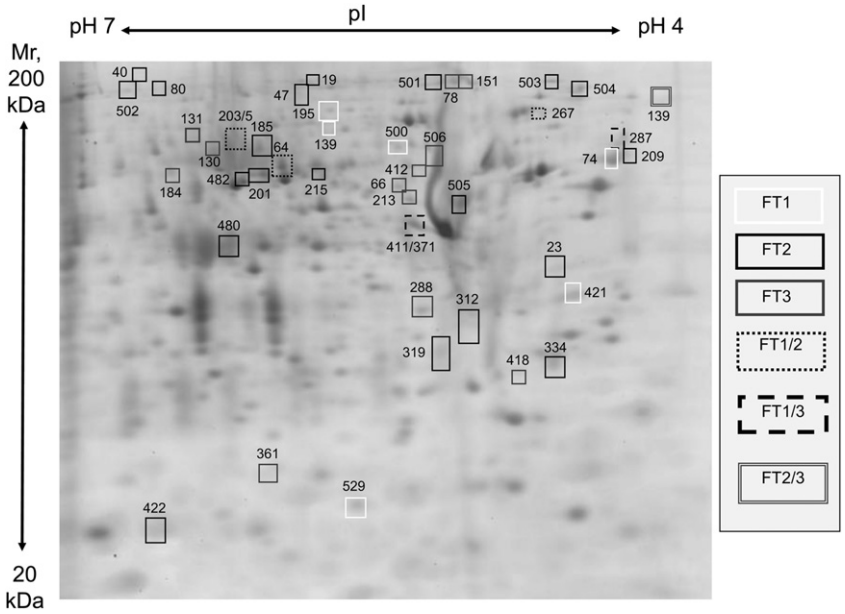

Fig. 2. A 2-DE gel showing the positions and numbers of protein spots that differed significantly in intensity between control and contaminated sites in each field trial, as well as spots that differed in intensity between control and contaminated sites in more than one field trial. FT1, FT2, FT3 = field trial 1, field trial 2 and field trial 3 respectively.

Table 2
Putative identifications and fold differences (relative to control) of proteins that were significantly different in intensity between control and contaminated sites.

Field trial	Spot #	Fold change	Number of peptides	Log(e)+ value	Putative identifications	Identity of proteins in NCBI database
FT1	74	−1.5	5	−127.3	Tropomyosin	gi|15419048| *C. gigas*
	500	1.4	18	−173.8	Actin	gi|229472804| *C. gigas*
	529	−2.7	5 and 4	−34.2 and −25.3	Vitellogenin and NADH dehydrogenase subunit 2	gi|168740395| *Pecten maximus* and gi|6048558| *M. californianus*
FT2	47	−1.9	4	−22.8	Vasa	gi|91179150| *Chlamys farreri*
	201	1.6	5	−33.4	Alpha-macroglobulin	gi|194293520| *Cristaria plicata*
	209	−2.2	5	−118.6	Tropomyosin	gi|15419048| *C. gigas*
	215	1.4	5	−50.2	Actin	gi|32816054| *C. farreri*
	312	1.5	5	−43	Sarcoplasmic calcium-binding protein	gi|6692814| *Mizuhopecten yessoensis*
	480	1.4	4	−32.7	Cytosolic malate dehydrogenase	gi|73656362| *M. californianus*
	482	1.3	4	−24.4 and −24.1	Ubiquitin-protein ligase and HSP70	gi|7524150| *Mya arenaria* and gi|92430370| *C. farreri*
FT3	66	2.8	74	−885.8	Actin 2	gi|18565104| *C. gigas*
	130	−2.2	5	−30.4	Sodium/glucose co-transporter	gi|48476121| *C. gigas*
	213	−2.5	40 and 7	−466.1 and −42.3	Actin 2 and HSP70	gi|18565104| *C. gigas* and gi|57635269| *M. galloprovincialis*
	288	−1.3	4	−30.5	Rho-GDI related protein	gi|33337635| *C. gigas*
	412	−1.8	14	−147.3	Beta-actin	gi|159507454| *Saccostrea kegaki*
	506	−2.0	19, 11, 10 and 9	−198.6, −109.7, −84.6, −78.4	Beta-tubulin, Extracellular SOD, Actin and Tubulin	gi|194068375| *S. kegaki*, gi|229485195| *S. glomerata*, gi|2833326| and gi|58219310| *C. gigas*
FT1/2	64	1.8	4	−23.7	Cytochrome *c* oxidase subunit 1	gi|27126468| *Pyganodon fragilis*
	267	−2.2	8	79.1	78 kDa glucose regulated protein (also known as HSP70)	gi|46359618| *C. gigas*
FT1/3	287	1.5	5	−45.4	Tropomyosin	gi|15419048| *C. gigas*
FT2/3	139	1.8	10	−95	Myosin heavy chain	gi|6682319| *M. galloprovincialis*

more than one field trial). Details of these putative identifications are shown in Table 2. Three of the identified proteins (which were identified in more than one protein spot) responded to all three field trials whilst several responded to just one field trial. Actin, tropomyosin and HSP70 were found to be differentially expressed between control and contaminated sites in all field trials. Vitellogenin and NADH dehydrogenase subunit 2 only responded during FT1. Vasa, alpha 2- macroglobulin, cytosolic malate dehydrogenase, ubiquitin-protein ligase and sarcoplasmic calcium-binding protein varied during FT2. Sodium/glucose co-transporter, Rho-GDI related protein and beta-tubulin were all identified in protein spots affected by FT3.

3.5. Biological functional categories

The identified proteins were assigned to one of 8 biological functional categories based on annotations from the NCBI database. Cytoskeletal activity (45% of proteins) was the most abundant biological function, followed by stress response and metabolism, which both accounted for a further 11% of the identified proteins (Fig. 4). Proteins associated with both cytoskeletal activity and stress response were found to vary during all three field trials.

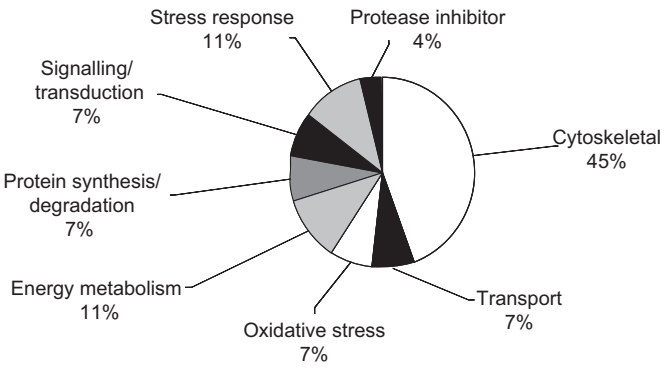

Fig. 4. Biological functions associated with the differentially expressed proteins identified by MS. $n = 20$.

3.6. Metal concentrations

Results of metal analyses are shown in Table 3. Ca, Pb and Zn in SPM differed significantly ($p < 0.05$) in concentration between control sites and contaminated sites in all three field trials. Al and Cd were also significantly more abundant in SPM at contaminated sites compared to control sites in FT3. During FT2, Cu and Mn were significantly higher in the SPM at contaminated sites.

In sediment, again both Pb and Zn were more abundant in contaminated sites relative to controls sites in all field trials. Cu, Cr, Fe, Mn and Ni also appeared at greater concentrations in contaminated sites during FT2.

There were fewer metals that differed in concentration in the dissolved fraction. Only Pb differed significantly between control and contaminated sites during FT1, and only Cd in FT3. Again, FT2 had the greatest number of metals (Cd, Cu, Pb and Zn) that were at higher concentrations in contaminated sites.

3.7. Principal components analysis

Fig. 5(A, B, C) shows the separation of metals in sediment, SPM and in the dissolved fraction respectively between control and contaminated sites over the three field trials. The concentrations of metals in sediment show a clear distinction between control and contaminated sites but does not show clear separation between field trials, except for contaminated sites in FT2. Metals in SPM show spatial and temporal separation with clear distinctions evident between the three field trials and between treatments. Metals in the dissolved phase also showed substantial temporal variability between field trials, but relatively less separation between contaminated and control sites.

Fig. 6 depicts the separation of the protein data over the three field trials revealing a clear distinction between each trial.

Fig. 7(A–C) shows the potential influence of metals and physical–chemical data on the protein patterns in FT1. All three PCA plots suggest that there is clear spatial separation between the locations of both the control sites and contaminated sites. In sediment (Fig. 7A) Cd, Cr and Ni correlated with the PC1 axis. The

Table 3
Average metal concentrations (ppm) in sediment, SPM and dissolved phase during three field trials (FT1, FT2, FT3) in Lake Macquarie. ±, standard deviation; *, significantly different (<0.05) levels of metals in contaminated sites compared to control sites; ns, no significant difference between contaminated sites compared to control sites.

	Al	Ca	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
FT1										
Sediment										
Control	29,183.13	8471.57	3.03	27.68	90.22	32,509.11	343.78	19.02	42.35	222.25
SD	± 1425.16	± 6748.14	± 2.61	± 5.72	± 19.12	± 2674.01	± 227.05	± 3.42	± 5.01	± 21.22
Contaminated	21,231.99	2037.92	15.28	17.17	180.41	29,238.46	247.05	8.67	739.50	1747.06
SD	± 4292.06	± 797.08	± 5.36	± 3.39	± 159.71	± 7607.54	± 163.63	± 2.62	± 470.49	± 1182.99
Sig.	ns	ns	*	ns	ns	ns	ns	*	*	*
SPM										
Control	17,602.01	17,962.86	0.80	18.37	65.43	18,774.87	897.80	13.10	25.02	180.70
SD	± 9830.93	± 3464.89	± 0.10	± 6.74	± 10.14	± 11,027.79	± 161.18	± 161.18	± 14.72	± 9.38
Contaminated	26,444.07	3263.37	6.38	21.15	144.23	27,875.79	1684.57	10.46	649.70	1114.35
SD	± 3479.76	± 408.50	± 5.61	± 3.53	± 96.38	± 2888.12	± 897.88	± 0.90	± 302.80	± 446.77
Sig.	ns	*	ns	ns	ns	ns	ns	ns	*	*
Dissolved										
Control			0.02		0.15				0.09	0.36
SD			± 0.01		± 0.02				± 0.00	± 0.02
Contaminated			0.03		0.16				0.13	0.93
SD			± 0.01		± 0.10				± 0.07	± 0.75
Sig.			ns		ns				*	ns
FT2										
Sediment										
Control	28,623.13	1817.46	1.18	19.25	71.74	23,227.16	227.28	10.99	29.72	127.77
SD	± 10,955.11	± 766.48	± 1.43	± 6.30	± 38.40	± 8556.53	± 142.07	± 4.08	± 4.74	± 24.63
Contaminated	31,448.88	5343.72	8.59	36.48	2238.43	92,525.14	5924.97	39.38	8810.50	5405.43
SD	± 11,851.38	± 4502.66	± 7.6	± 14.93	± 727.93	± 46,149.25	± 4884.72	± 9.31	± 6001.76	± 3822.21
Sig.	ns	ns	ns	*	*	*	*	*	*	*
SPM										
Control	26,990.44	1786.50	1.25	26.56	100.55	26,836.01	533.74	17.85	37.74	218.48
SD	± 3571.70	± 10,712.35	± 0.45	± 4.80	± 35.92	± 2369.72	± 277.99	± 4.25	± 5.16	± 33.59
Contaminated	24,410.82	7973.49	2.75	20.21	466.50	32,879.46	2124.78	15.37	1601.82	3049.28
SD	± 4343.05	± 1996.46	± 0.57	± 4.16	± 207.14	± 1790.08	± 757.87	± 4.04	± 41.47	± 541.97
Sig.	ns	*	ns	ns	*	ns	*	ns	*	*
Dissolved										
Control			0.02		0.17				0.09	0.94
SD			± 0.00		± 0.08				± 0.00	± 0.43
Contaminated			0.05		0.37				0.29	4.3
SD			± 0.00		± 0.12				± 0.10	± 0.70
Sig.			*		*				ns	*
FT3										
Sediment										
Control	33,244.66	9806.67	3.49	31.06	102.81	36,451.25	386.56	18.49	37.90	179.50
SD	± 1187.49	± 7985.21	± 3.09	± 6.13	± 22.03	± 3890.40	± 245.18	± 5.25	± 5.39	± 60.67
Contaminated	24,671.67	2373.05	17.73	19.95	205.19	33,947.80	285.86	10.06	982.96	1355.53
SD	± 4633.10	± 903.45	± 6.02	± 3.64	± 176.15	± 8375.81	± 184.95	± 2.88	± 221.46	± 958.50
Sig.	ns	ns	*	ns	ns	ns	ns	ns	*	*
SPM										
Control	20,301.56	20,530.72	0.91	20.73	74.35	21,246.98	1019.80	14.90	28.87	203.64
SD	± 11,678.44	± 4448.58	± 0.09	± 7.89	± 9.08	± 12,698.84	± 149.87	± 1.36	± 17.51	± 12.73
Contaminated	30,743.97	3807.71	7.45	24.59	164.74	32,416.32	1967.16	12.17	787.20	1273.15
SD	± 3652.91	± 454.88	± 6.55	± 3.89	± 105.27	± 2889.69	± 1072.23	± 0.86	± 361.67	± 507.08
Sig.	*	*	*	ns	ns	ns	ns	ns	*	*
Dissolved										
Control			0.01		0.09				0.09	0.45
SD			± 0.00		± 0.00				± 0.00	± 0.14
Contaminated			0.05		0.13				0.11	0.97
SD			± 0.01		± 0.03				± 0.03	± 0.03
Sig.			*		ns				ns	ns

protein expression at control site 2 was associated with Cr and Ni, whereas the contaminated sites were more influenced by Cd, Cu, Pb and Zn. Ca, Fe and Al correlated with PC1 axis in SPM (Fig. 7B). Ca influenced the protein expression at control site 2 whilst Al, Cd, Cr, Fe, Pb and Zn were related to the protein concentrations in oysters from contaminated sites. Cu, Pb and Zn in the dissolved phase, as well as pH, influenced the protein expression patterns of oyster haemolymph at contaminated sites, whilst salinity appeared to have the greatest influence on proteomes at control site 2 (Fig. 7C).

Fig. 7(D–F) shows the relationships between metals and physical–chemical data on the differentially expressed proteins in FT2. Overall there is less spatial separation between the sites and locations in this field trial. Metals in the sediment did not appear to influence the proteins patterns during this field trial. In contrast, Ca, Cr and Al in the SPM (Fig. 7E), along with salinity and pH (Fig. 7F), influenced protein expression at the majority of the sites.

Although separation was evident in the combined PCA analysis between control and contaminated sites in FT3, there was also more variability within sites (Fig. 7G–I). A suite of metals in the sediment corresponded with protein expression, with Cu and Mn affecting contaminated sites and Al, Mn and Ni relating to differential proteins at the control sites. Mn in SPM was associated with the concentration of proteins in oysters from the contaminated sites, whilst protein expression at control sites did not appear to be

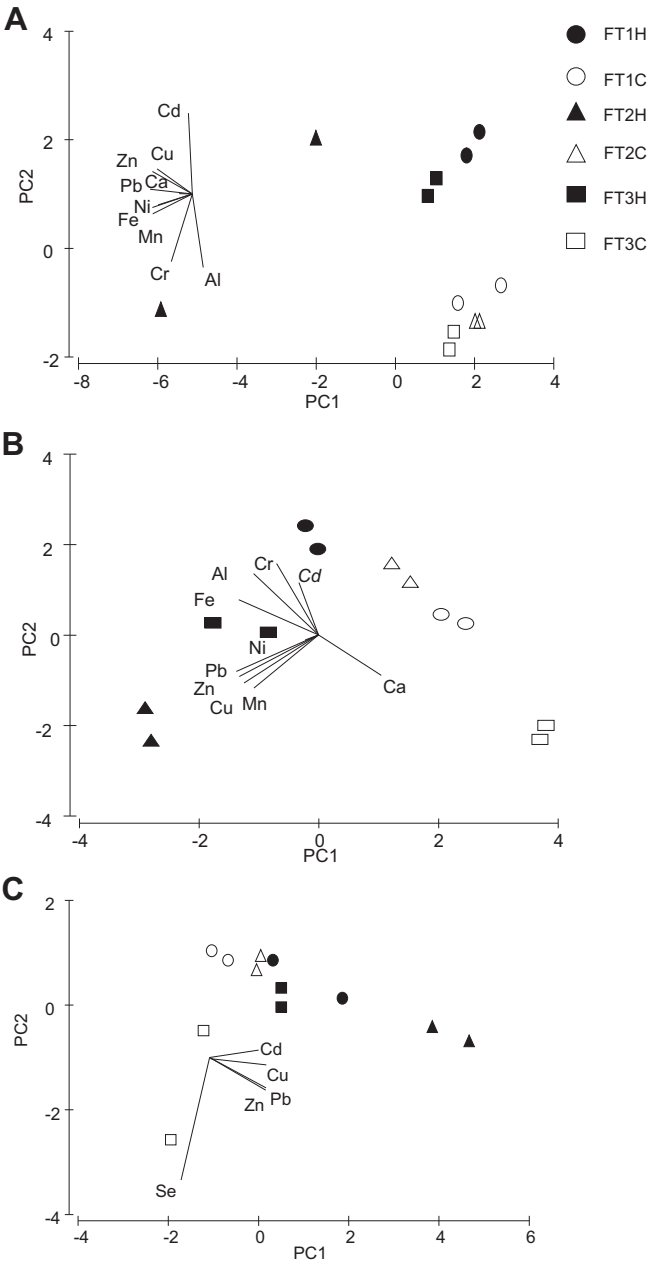

Fig. 5. (A–C). PCA analysis showing the spatial and temporal distribution of metals in sediment (5A), SPM (5B) and dissolved phase (5C) respectively, per field site, per field trial. FT1, 2, 3 = field trials 1, 2 and 3 respectively. C = control sites. H = contaminated sites.

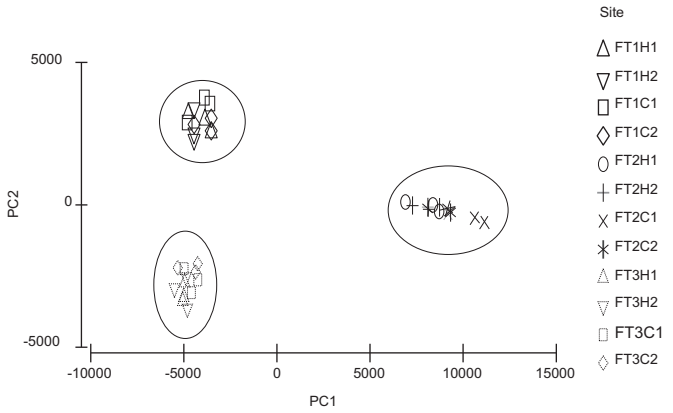

Fig. 6. PCA analysis showing the spatial and temporal separation of protein spot abundances per field sites, per field trial. FT1, 2, 3 = field trials 1, 2 and 3 respectively. C = control sites. H = contaminated sites.

influenced by metals in SPM. In the dissolved phase, metal concentrations did not appear to be associated with protein concentration in either treatments. Instead, pH influenced the proteomes of oysters at contaminated sites and salinity was related to changes in protein expression at the control sites.

4. Discussion

Metal contamination in estuarine waterways can have severe impacts on a range of organisms from the cellular to ecological levels. For instance, Roach et al. (2001) found differences in the species composition of ecosystems where contaminants were found in significantly different levels (ie. Cd ranging from 2 to 10 mg/kg, Cu from 20 to 400 mg/kg, Pb from 50 to 1080 mg/kg and Zn from 100 to 2200 mg/kg). At a cellular level Vlahogianni et al. (2007) found changes in levels of oxidative stress in *M. galloprovincialis* after exposure to various heavy metals, including Cd, Cu, Pb and Zn all at under 5 μg/l. Similarly, changes in protein expression have been detected in *S. glomerata* after exposure to Cd, Cu, Pb and Zn at concentrations from 5 to 100 μg/l in the laboratory (Thompson et al., 2012). These changes in oyster proteomes under laboratory conditions led us to investigate the proteomes of the Sydney Rock oyster that had been transplanted to sites of contamination and control areas (non-impacted) in Lake Macquarie, NSW, to assess the efficacy of proteomics as a monitoring tool in the field.

The current study showed that exposing Sydney Rock oysters to different levels of contamination and other environmental variables (predominantly salinity and pH) in the field has significant impacts on the proteomes of oyster haemolymph. For each of the three field trials undertaken in the study, 2-DE analysis identified a distinct set of protein spots that differed in intensity in oysters from the contaminated sites when compared to control sites. There was also some overlap in the differential protein spots between the field trials, but none of the differential protein spots changed in response to all three trials (even though three proteins identified by mass spectrometry, actin, tropomyosin and HSP70, which were found in more than one protein spot, did appear to be common in all three field trials). This suggests that impacts on protein expression are most likely due to the observed variation in the combinations of contaminants or other environmental variables over time. Data suggest that salinity and, to a lesser extent, pH have significant effects on oyster proteomes in conjunction with metals. A previous study by Apraiz et al. (2009) also that found that the sets of differential proteins identified by 2-DE varied spatially and temporally in the mussel, *Mytilus galloprovincialis,* in response to oil contamination. This suggests that whilst proteomics can routinely detect differences between contaminated and non-contaminated treatment areas, it is also sensitive enough to identify changes in the mixture of specific contaminants combined with other environmental variables over time.

The temporal variability identified in the current study brings into question field-based proteomic analyses that focus on just one time point. Such studies include the identification of differentially expressed proteins in the mussel, *M. edulis*, at field sites in Norway containing metal and PAH contamination (Knigge et al., 2004), the effects of metal and pesticide contamination on proteins in the crab, *C. maenas*, in the Domingo Rubio stream, Spain (Montes Nieto et al., 2010), the differential expression of peroxisomal proteins in

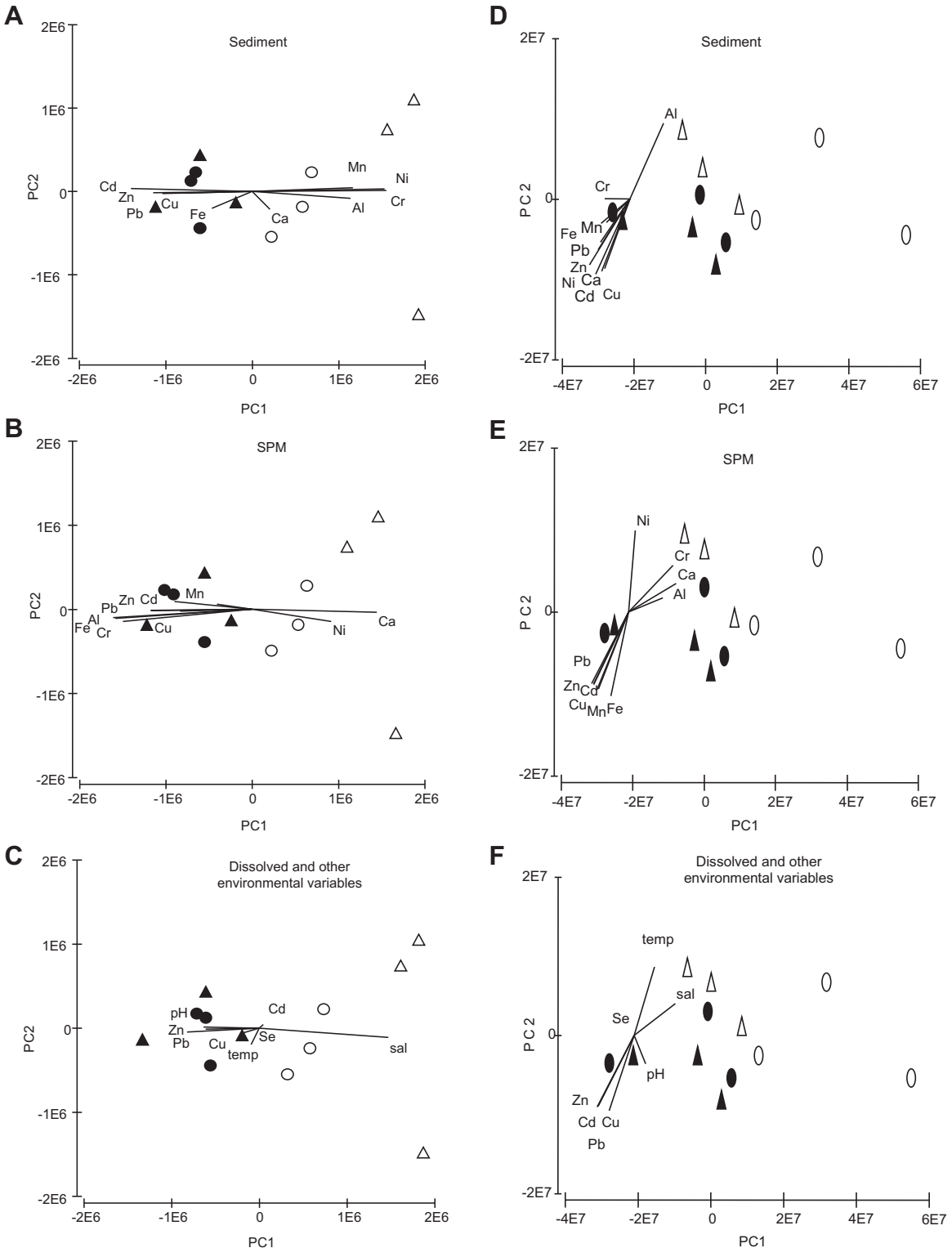

Fig. 7. (A–I). PCA analysis showing the influence of metals in sediment, SPM and dissolved phase/physical chemical data respectively in FT1 (A–C), FT2 (D–F), and FT3 (G–I). ○ FT1 C, △ FT2 C, □ FT3 C, ● FT1 H, ▲ FT2 H, ■ FT3 H.

the mussel, *M. galloprovincialis*, at reference compared to contaminated sites linked to organic xenobiotics (Mi et al., 2005), and changes in protein expression patterns of the mussel, *M. edulis*, in response to marine pollution in Gothernburg Harbour, Sweden (Amelina et al., 2007). Even though these single time point studies have detected changes in protein expression patterns in areas with different contamination levels, our work highlights the importance of analyzing multiple time periods in order to develop a broader picture of the range of effects possible in the field.

There are a number of possible explanations for the different proteome patterns seen between the three field trials in the current study. The first is that the combinations and concentrations of

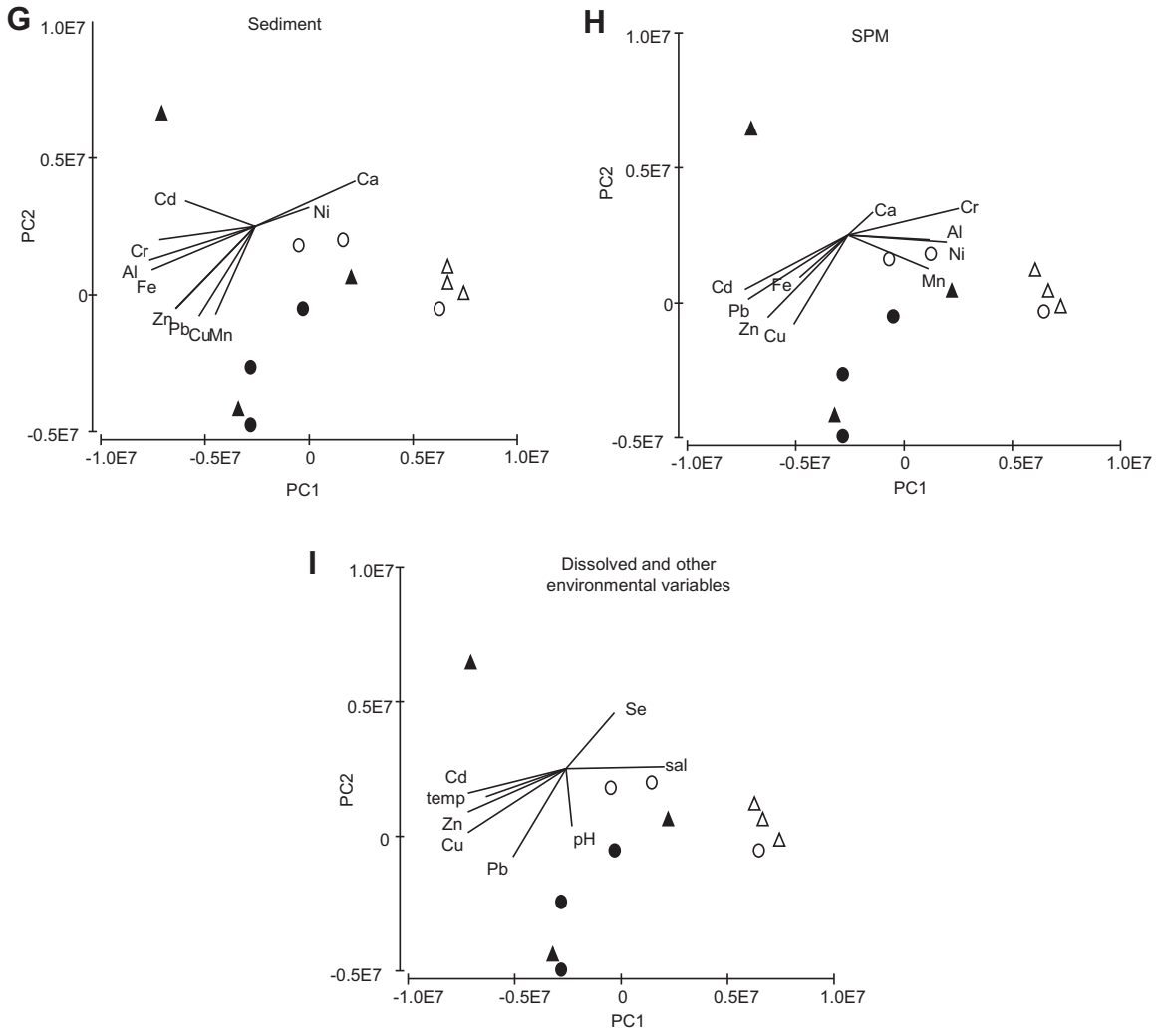

Fig. 7. (*continued*).

contaminants varied substantially between the three field trials. For instance, Pb and Zn levels varied substantially throughout the trials (649 mg/kg, 1601 mg/kg and 787 mg/kg Pb, and 1114 mg/kg, 3049 mg/kg and 1273 Zn in FT1, FT2 and FT3 respectively). In laboratory-based exposures using individual metals, proteomic studies have shown that even small changes in metal concentrations (for instance 5 μg/l) elicit different protein expression patterns (Rodríguez-Ortega et al., 2003; Son et al., 2011; Thompson et al., 2011). The greatest number of differentially expressed proteins identified between contaminated and control areas was during FT2. Interestingly, this field trial also had the greatest number of different metals that changed significantly in concentration between the two areas (8 metals across the three fractions tested). This suggests that the synergistic effects, or the number of metals, may have increased impacts on the *S. glomerata* proteome. The levels of contaminants observed in Lake Macquarie in this study have been shown to have an impact on the Sydney Rock oyster at the cellular level affecting a range of proteins. Similar concentrations of metals in previous studies have also been shown to impact on oysters from at population and ecosystem levels (Roach et al., 2001) suggesting that changes seen in this study may have ongoing consequences.

PCA analysis allowed us to independently assess the association of a range of metals in the sediment, SPM, dissolved fractions, as well as other environmental variables, with oyster proteomes at each field trial. The analysis showed that in each field trial a different suite of metals, combined with salinity and pH influenced the proteome patterns of oysters. A suite of metals were associated with differential protein expression during FT1. These included Cd, Pb and Zn in sediment, Cr, Pb and Zn in SPM and Cu and Pb in the dissolved phase. These metals were all associated with the contaminated sites and were all at significantly different concentrations between the control and contaminated sites. Salinity also had a significant influence on protein expression during FT1 at control sites whilst pH was associated with contaminated sites. During FT2 there was very little relation between proteomes and metals in sediment or in the dissolved phase. PCA suggests that just Ca and Cr in SPM were associated with protein patterns. Salinity and pH appeared to impact on both control and contaminated sites but to a lesser extent than in FT1. During FT3, Al, Cu, Mn and Ni influenced either the control or contaminated sites. Again salinity and pH were associated with protein expressions similar to those shown in FT1.

It is also unlikely that all of the contaminant fractions analysed had the same impact on the *S. glomerata* proteome due to differences in the bioavailability of metals in these fractions. The bioavailable component of metals dissolved in the water column is normally higher than that of metals on SPM (Knezovich, 1992).

However, there is a higher concentration of metals adsorbed to SPM than there is in the dissolved phase. In addition, bio-availability is substantially increased at the interface of salt and freshwater, as desorption is high due to high dilution rates, the presence of soluble anions in seawater competing for sorption of metals to form soluble complexes and the competitive exchange of inorganic cations on the sorptive substrate (Knezovich, 1992). Although desorbed metals are quickly re-adsorbed (the mathematical model by Orlob et al. (1980) suggests equilibria is reached after 5 h following a relatively large discharge of Cu) bioavailability is high where initial dilution occurs. Therefore, although metals are readily available for uptake by organisms in the dissolved phase, a number of studies suggest that metals adsorbed to SPM also contribute substantially to the overall uptake by organisms (Langston and Spence, 1995). The multivariate analyses indicated that metals in the dissolved phase during FT1 and metals in SPM in all field trials accounted for more of the variation in oyster proteomes than metals in the sediment, and interestingly that salinity and pH, which may also affect bioavailability (Knezovich, 1992), were also influential.

Given that the highest number of differentially expressed proteins between control and contaminated sites occurred during FT2, but PCA suggests metals had little influence, it is likely that other variables such as salinity and pH have substantial impacts on oyster proteomes. Although there were no significant differences in temperature, salinity and pH between control and contaminated sites during any field trial, PCA analysis suggests that both pH (predominantly at contaminated sites) and salinity (predominantly at control sites) did have an influence on the oyster proteome. Changes in salinity have been previously shown to impact on molluscs. Paterson et al. (2003) suggested that an increase in salinity reduces the growth rate of *S. glomerata*. At an enzymatic level, reduced salinity inhibits phenoloxidase levels in *S. glomerata* (Butt et al., 2006). Changes in salinity result in differential gene expression in *C. gigas* (Boutet et al., 2005). Changes in pH have also been shown to affect Sydney Rock oysters. Decreased pH can lead to reduced feeding ability and thus increased mortality (Dove and Sammut, 2007) and it has also been correlated with reduction in shell growth in *S. glomerata* larvae (Parker et al., 2011). At a molecular level, low pH has been shown to influence protein expression in *S. glomerata* larvae (Parker et al., 2012).

The identification of differentially expressed proteins by mass spectrometry in the current study suggests that there are a few highly represented proteins and associated biological functions that change in response to contaminants and other environmental variables. These include actin, which is involved in cytoskeletal activities, and HSP70 which is a stress response protein. These proteins responded in all of the three field trials (albeit in different protein spots on 2-DE gels). These proteins have also been shown to change in response to other contaminants in the field. Actin in the mussel *M. galloprovincialis* was found to change in response to oil in field trials (Apraiz et al., 2009), whilst HSP70 was differentially expressed between control and contaminated sites in *M edulis*, responding to marine pollution (Amelina et al., 2007) and to Cu (Sanders, 1993; Sanders and Steinert, 1996), and in proteomic field trials after arsenic exposure in the fathead minnow (*Pimephales promelas*) (Dyer et al., 1993). Interestingly, these proteins also responded in Sydney Rock oyster haemocytes during laboratory-based exposures to metals (Thompson et al., 2011, 2012).

Other proteins identified by mass spectrometry in the current study have also been previously associated with biological responses to contamination. These include beta-tubulin (Apraiz et al., 2009) and SOD (Rosa et al., 2005). Overall, our analysis suggest that proteins involved in the cytoskeleton and, to a lesser extent, energy metabolism and stress responses, were the most frequently affected by environmental variables in the field. The use of 2-DE has increased our knowledge of the effects of contaminants and environmental variables on the biological functions of the Sydney Rock oyster. Many of these biological functions can be analysed using fast throughput assays alleviating some of the need for 2-DE analysis.

In summary, the current study shows that proteomics has been extremely useful in linking the effects of contaminants and other environmental variables between contaminated and control sites in the field. Even though there were differences in the proteins differentially expressed during each trial, this does not rule out the use of proteomic biomarkers as a multifactorial tool including fast throughput assays to assess the biological impacts of environmental contamination and other physical–chemical parameters.

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