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Baseline

A comparative proteomic study on the effects of metal pollution in oysters *Crassostrea hongkongensis*

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ABSTRACT

The metal pollution has posed great risk on the coastal organisms along the Jiulongjiang Estuary in South China. In this work, two-dimensional electrophoresis-based proteomics was applied to the oysters *Crassostrea hongkongensis* from metal pollution sites to characterize the proteomic responses to metal pollution. Metal accumulation and proteomic responses indicated that the oysters from BJ site were more severely contaminated than those from FG site. Compared with those oyster samples from the clean site (JZ), metal pollution induced cellular injuries, oxidative and immune stresses in oyster hepatopancreas from both BJ and FG sites via differential metabolic pathways. In addition, metal pollution in BJ site induced disturbance in energy and lipid metabolisms in oysters. Results indicated that cathepsin L and ferritin GF1 might be the biomarkers of As and Fe in oyster *C. hongkongensis*, respectively. This study demonstrates that proteomics is a useful tool for investigating biological effects induced by metal pollution.

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Metal pollution in the estuarine and coastal environments in South China has posed a significant threat to coastal organisms (Luo et al., 2014; Wang et al., 2014). Previous researches reported that the oysters *Crassostrea hongkongensis* and *Crassostrea sikamea* from the metal-polluted sites along the Jiulongjiang Estuary, Fujian Province, were severely contaminated by copper, which was marked by the blue or green tissues in oysters with the high tissue Cu concentration up to 19,000 µg/g dry weight (Weng and Wang, 2014). Therefore, it is necessary to elucidate the biological effects induced by metal pollution, which may be further used to predict the consequence of metal pollution.

As a marine filter-feeder, oysters are not only edible bivalves but play an important role in maintaining the marine ecosystem health in many estuarine systems (Weng and Wang, 2014). Due to its high capacity to accumulate metals, oysters such as *Crassostrea hongkongensis*, *Crassostrea sikamea* and *Saccostrea glomerata* are also preferable environmental bioindicators for metals (Goldberg et al., 1983). Thompson et al. (2011) used Sydney rock oyster *Saccostrea glomerata* as the bioindicator to investigate the effects of metals including Cd, Cu, Zn and Pb, which suggested that this oyster was a good bioindicator of metal pollution. Evidences have indicated that the oyster *C. hongkongensis* distributed along the coast in South China is a hyperaccumulator of Cu and Zn (Tan et al., 2015). In this study,

therefore, the oyster *C. hongkongensis* was selected to investigate the biological effects of metal pollution.

Using toxicity-related biomarkers of metals, such as the anti-oxidative enzyme activities and metallothioneins, to monitor metal pollution has been accepted in ecotoxicology and environmental monitoring programs (Regoli, 2000; Rank et al., 2007). Among these established biomarkers, however, few of them have adequate sensitivity, specificity and predictability for metal pollution. With the rapid development of -omic techniques, researchers can carry out a global analysis on the molecules to obtain a set of biomarkers related to the biological effects induced by metal pollution, which facilitates the detection and prediction of metal pollution (Cappello et al., 2013; Knigge et al., 2004). Among these -omic techniques, proteomics can theoretically analyze all the proteins encoded by the given genome in an organism (Knigge et al., 2004). Therefore, a comparative proteomics may compare the whole protein profiles in the selected environmental bioindicators from normal and metal pollution-stressed conditions and present the proteomic differences induced by metal pollution. Based on its applicability in ecotoxicology and environmental biology, comparative proteomics has been used as a diagnostic tool for environmental pollution assessment (Campos et al., 2012).

In this study, we applied two-dimensional electrophoresis (2-DE)-based proteomics to investigate the proteomic responses in oyster (*Crassostrea hongkongensis*) to metal pollution. The oysters *C. hongkongensis* were collected from three sites (Baijiao, Fugong and

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Jiuzhen) along the Jiulongjiang Estuary, Fujian Province, China. Among these sampling sites, the former two sites were contaminated by different concentrations of metals (such as copper, zinc, cadmium and iron), respectively. The Jiuzhen site was a relatively clean and used as a reference. The hepatopancreas tissues of oysters were collected for metal analysis and proteomics, as the hepatopancreas tissue is the main storage and detoxification organ for metals (Jones et al., 2008). The aims of this study were to characterize the proteomic responses and biological effects in oysters *C. hongkongensis* exposed to metal pollution using 2-DE-based proteomics.

During the low tides, the contaminated oysters *C. hongkongensis* were collected from Baijiao (BJ, 24°28'2" N, 117°56'19" E) and Fugong (FG, 24°22'58" N, 117°54'13" E) sites along the contaminated Jiulongjiang Estuary, and the control oysters were collected from the harbor of Jiuzhen (JZ, 24°2'38" N, 117°42'26" E) site from the nearby relatively clean Jiuzhen estuary, Fujian Province, in December 2012 (Fig. 1). This estuary was an important area for oyster culture, and the average surface salinity ranged from 14 to 26 psu as for tidal actions (Liu and Wang, 2012). Ten individual oysters with similar sizes were sampled from each site, and the tissues of hepatopancreas were immediately dissected and flash frozen in liquid N₂. After transported to the laboratory, the oyster samples were stored at –80 °C before protein extraction and metal determination. All the practical procedures for oyster sampling were strictly performed according to the guidelines suggested by Hines et al. (2007).

Total protein extraction was performed based on previous studies with some modifications (Wu et al., 2013a). Briefly, the oyster samples were homogenized quickly on ice with 1 mL of TRIzol reagent and centrifuged at 12,000g for 5 min at 4 °C. The supernatant was added with 200 µL of chloroform before shaking vigorously for 3 min and precipitating for 3 min. The mixture was centrifuged at 12,000g for 15 min at 4 °C, and its upper aqueous layer was discarded. A volume of 300 µL of absolute ethyl alcohol was added and the mixture was allowed to stand for 3 min at room temperature before being centrifuged at 2000g for 5 min at 4 °C. The phenol/ethanol supernatant was precipitated for 30 min at room temperature by the addition of 750 µL of isopropanol

prior to centrifugation at 14,000g for 10 min at 4 °C. The pellets were washed with 1 mL of ethanol (v/v 95%) and centrifuged at 14,000g for 10 min at 4 °C. This procedure was repeated twice. The pellets were solubilized in the lysis buffer (7 M urea; 2 M thiourea; 4% m/v CHAPS; 65 mM DTT and 0.2% W/V Bio-lyte buffer) and then incubated for 3 h at room temperature. The homogenate was centrifuged at 15,000g for 10 min and the supernatant was applied to electrophoresis. The total concentrations of proteins were determined by Protein Assay Kit of TianGen.

For the first dimension (IEF), 130 µg of protein was loaded onto IPG strips with a linear pH gradient from 4 to 7 (Immobiline Drystrip TM 24 cm, GE Healthcare, USA) The isoelectric focusing gel solution (7 M urea, 2 M thiourea, 4% m/v CHAPS, 65 mM DTT, 0.001% m/v bromophenol blue and 0.2% W/V Bio-lyte buffer). IEF was conducted at 20 °C with an Ettan IPGphor3 system for a total of 85,858 Vh (active rehydration was carried out at 30 V for 12 h, followed by 100 V for 5 h, 500 V for 1 h, 1000 V for 1 h, and a linear increase of voltage to 8000 V for 11 h and stand by 500 V for the second dimension). After the first dimension, all the strips were placed in equilibration buffer (0.05 M Tris–HCl, pH 8.8; 6 M urea; 30% glycerol; 2% (w/v) SDS; containing 1% (w/v) DTT) and were slowly shaken for 15 min. The strips were then incubated for another 15 min in the equilibration buffer with 2.5% (w/v) iodoacetamide without DTT. The second dimension was conducted on 12.5% SDS-PAGE gels using the Ettan DALTSix system. After electrophoresis, the gels were silver stained by following the method of Mortz et al. (2001) and Gharahdaghi et al. (1999). Images were captured by ImageScanner III and spots were quantitatively analyzed using ImageMaster 2D Platinum 7.0. For all the matched spots, only protein spots with significant changes of at least 1.5-fold, and deemed significant by Student's *t*-test at a level of 95% were accepted as differentially expressed proteins.

In gel digestion was performed according to Katayama et al. (2001). After being completely dried, the samples were re-suspended with 5 µL of 0.1% TFA followed by mixing in 1:1 ratio with a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile (Shevchenko et al., 1996). One microliter of mixture was analyzed by



Fig. 1. The map showing the locations of sampling sites along the Jiulongjiang Estuary, Fujian Province, China. Jiuzhen: 24°2'38" N, 117°42'26" E; Baijiao: 24°28'2" N, 117°56'19" E; Fugong: 24°22'58" N, 117°54'13" E.

Table 1

Metal/metalloid concentrations in hepatopancreas tissues from oysters *Crassostrea hongkongensis* sampled from three estuarine sites (JZ, BJ and FG).

Metal/metalloid concentration ($\mu\text{g/g dw}$) ^a	Sampling site		
	JZ	BJ	FG
Cr	0.5 \pm 0.2	18.4 \pm 1.7**	17.3 \pm 2.7**
Mn	23.8 \pm 11.6	65.0 \pm 36.1	50.9 \pm 27.4
Fe	302.6 \pm 158.9	1821.5 \pm 993.0**	550.3 \pm 181.6
Co	0.7 \pm 0.3	3.0 \pm 0.3**	1.4 \pm 0.3*
Ni	1.3 \pm 0.6	11.4 \pm 0.8**	7.6 \pm 2.2**
Cu	207.9 \pm 83.0	5052.7 \pm 1961.7**	2516.0 \pm 1738.6**
Zn	3972.3 \pm 862.3	8015.7 \pm 3146.0*	7739.0 \pm 4081.1
As	11.8 \pm 1.8	26.7 \pm 2.4**	15.3 \pm 3.4
Ag	1.3 \pm 0.6	9.0 \pm 2.8**	2.2 \pm 1.1
Cd	4.7 \pm 0.7	18.2 \pm 1.6**	23.4 \pm 5.3**
Pb	2.2 \pm 0.4	5.4 \pm 1.2**	3.8 \pm 0.7**

* ($P < 0.05$) and ** ($P < 0.01$) mean the significant differences of metal concentrations between clean (JZ) and metal pollution sites (BJ or FG) (Student's *t*-test).

^a Data are shown as mean \pm standard deviation ($n = 5$). Values are presented as $\mu\text{g/g}$ dry weight.

an ABI 4800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, USA), data were acquired in a positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI4800 Calibration Mixture). Both the MS and MS/MS data were integrated and processed using the GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters. Proteins were successfully identified based on 95% or higher confidence interval of their scores in the MASCOT V2.4 search engine (Matrix Science Ltd., London, U.K.). The following parameters were used in the search: NCBI nr Metazoa (Animals) (2,861,494 sequences) database; trypsin as the digestion enzyme; one missed cleavage site; partial modifications of cysteine carbamidomethylation and methionine oxidation; no fixed modifications; 0.15 Da for precursor ion tolerance and 0.25 Da for fragment ion tolerance. Individual ions scores > 40 indicate identity or extensive homology ($P < 0.05$).

The oyster tissue samples were dried to the constant weights and then digested in concentrated HNO_3 at 80 °C for 12 h, after which a clear liquid was obtained. Metal concentrations in the samples were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x). Appropriate internal standards (Sc, Ge, In, Bi) were selected to correct the sensitivity drift and matrix effect. A quality control sample was repeatedly measured after every 10 samples. The recovery of the analyzed metals from the standard reference material (SRM 1566b, oyster tissue) was within 10% deviation from the certified values except Cr, of which the certified concentration was not available. Metal

concentrations were expressed as mean \pm standard deviation (S.D.). All the data of metal concentrations were subjected to principal component analysis with autoscaling and Student's *t*-test. A *P* value < 0.05 was considered significant. Statistical analysis was performed using Minitab software (Version 15, Minitab Inc. PA, USA).

The hepatopancreas tissue of marine bivalves is the most important organ for metal accumulation and detoxification. The concentrations ($\mu\text{g/g}$ dry weight) of ten metals (Cr, Mn, Fe, Co, Ni, Cu, Zn, Ag, Cd and Pb) and one metalloid (As) in hepatopancreas from the oysters *C. hongkongensis* collected from clean (JZ) and metal contaminated (BJ and FG) sites are summarized in Table 1. To discern metal contamination situations, principal component analysis (PCA) were conducted to summarize the differences between different sampling sites, using the metal/metalloid concentrations as variables (Fig. 2). The biplot containing samples (oyster samples from the sampling sites) and variables (ten metals and one metalloid) is shown in Fig. 2, with the first two principal components (PCs) interpreting 81.6% of the variance from the original data set of metal concentrations in oyster hepatopancreas tissues. Visibly, these three groups of samples from JZ, BJ and FG sites were clearly separated along PC1 axis, which demonstrated the significant differences of tissue metal concentrations between the samples from the three sampling sites. Overall, along PC1 axis, the oyster samples from both BJ and FG contained higher average levels of Cr, Co, Ni and Cu than those from JZ site. Specifically, the metals/metalloid including Cu, Fe, Co, Pb, Ag and As, were distributed in the cluster of samples from BJ site, suggesting that the samples from BJ site contained the highest average concentrations of these six metals/metalloid, with statistical significances (Table 1). The samples from FG contained the highest concentrations of Cd as shown in Table 1.

Simply to compare the severity of metal pollution, an equation suggested by Liu and Wang (2012) was used to evaluate the integrated metal contamination:

$$\text{Integrated metal contamination} = \sum_{i=0}^m C_{\text{contaminated}}^i - C_{\text{clean}}^i$$

where $C_{\text{contaminated}}^i$ is the concentration of the *i*th metal/metalloid in a contaminated oyster, and C_{clean}^i is a reference value for the *i*th metal/metalloid in an uncontaminated one. The reference was determined from the clean JZ oysters. And *m* is the number of all metals/metalloid measured in oyster tissue samples, i.e., $m = 11$ in this work. Calculation indicated that the oysters sampled from BJ site were the most severely contaminated ones because of the massive contribution from the very high tissue concentrations of Cu and Fe in oyster hepatopancreas.

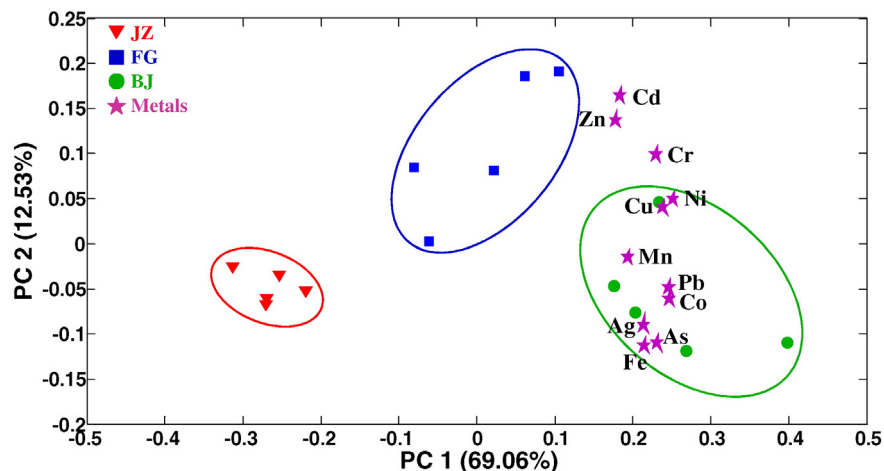


Fig. 2. The biplot containing the PC scores for oyster samples from the three sampling sites, Jiuzhen (JZ, \blacktriangledown), Baijiao (BJ, \bullet) and Fugong (FG, \blacksquare) and the variable (eleven metals/metalloid, \star) contributions for the clustering of oyster samples.

Proteomic responses induced by metal pollution were determined by 2-DE-based proteomics in oyster hepatopancreas (Fig. 1). Approximately, 1000 protein spots were resolved in the 2-DE gels from the hepatopancreas of oysters *C. hongkongensis*. A total of 24 spots resolved in 2-DE gels were differentially expressed (>1.5 folds, $P < 0.05$) in the oyster samples from both BJ and FG sites. Fig. 1 shows the differential protein spots in hepatopancreas of oyster *C. hongkongensis* sampled from BJ and FG sites compared with those from the reference site, JZ. All the differentially expressed proteins in all three biological replicates of silver-stained gels were analyzed by MALDI-TOF/TOF mass spectrometry. The information of these proteins is summarized in Table 2.

In the oyster samples from BJ site, 22 proteins were differentially expressed, including 19 down-regulated and 3 up-regulated proteins. These proteins were basically related to oxidation-reduction process, metabolism, cytoskeleton, protein synthesis, chaperones, ion homeostasis, antioxidant system, signal transduction and transport. Only 7 proteins were significantly differentially expressed in the oyster samples from FG site, including 4 down-regulated and 3 up-regulated proteins. Among these 7 proteins, 5 proteins (SH3 domain-binding glutamic acid-rich-like protein 3, cofilin, actin, 78 kDa glucose regulated protein and mammalian ependymin-related protein 1) were common in the oyster samples from both BJ and FG sites.

SH3 domain-binding glutamic acid-rich-like protein belongs to a new family of highly conserved small proteins related to thioredoxin superfamily which is involved in the control of redox dependent processes (Mazzocco et al., 2002). The structural features of alpha-crystallin B suggest that this protein is a small stress protein related to small heat shock protein family, while the 78 kDa glucose regulated protein is another stress protein belonging to the 70 kDa heat shock protein family. Evidences indicated that the heat shock proteins are responsive to heavy metal-induced oxidative stress (Fontaine et al., 2003). Peroxiredoxin, an antioxidant enzyme reducing hydrogen peroxide (H_2O_2) and alkyl hydroperoxides treatments, regulates peroxide-mediated signaling cascades, while superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Fujii and Ikeda, 2002). These two proteins are also responsive to oxidative stress. Cytochrome b5 is involved in the cytochrome P450 monooxygenase reaction which is related to the oxidative stress induced by xenobiotics, such as Cd and Cu (Zhang et al., 2012). These 6 altered antioxidant proteins indicated the oxidative stress induced by metal pollution in oysters *C. hongkongensis* from BJ site. Among these 6 proteins, only SH3 domain-binding glutamic acid-rich-like protein and 78 kDa glucose regulated protein were similarly altered in the oyster samples from FG site, which suggested that the metal pollution in FG site induced less severe oxidative stress in oysters. This finding is consistent with the less severe metal pollution in FG site, as mentioned above.

Actin is an abundant cytoskeletal protein that is a component of microfilaments in cells (Puerto et al., 2011). Both F-actin-capping protein and twinfilin are actin-binding proteins that are involved in the regulation of actin dynamics (Hartmann et al., 1989; Matzavinos and Othmer, 2007). Actin-depolymerizing factor is one of the actin cytoskeleton-modulating proteins, while cofilin promotes actin polymerization and defines the direction of cell motility (Allen et al., 1997; Ghosh et al., 2004). These proteins are all directly involved in cytoskeletal structure. Myosin regulatory light chains are regulators in the myosin contractile activity related to muscle contraction (Serwe et al., 1993). The tubulin-specific chaperones function as a tubulin assembly machine, marrying the α - and β -tubulin subunits into a tightly associated heterodimer (Tian and Cowan, 2013). The significant alterations of these seven cytoskeleton-related proteins (actin, F-actin-capping protein, twinfilin, actin-depolymerizing factor, cofilin, myosin regulatory light chains and tubulin-specific chaperone A) confirmed the cellular injury in oysters induced by metal pollution in BJ site. In numerous previous studies, some of these proteins such as actin, actin-depolymerizing factor, myosin regulatory light chain and F-actin-capping protein, were responsive to environmental stressor (e.g., arsenic, salinity, tetrabromobisphenol

A)-induced oxidative stress in animals (Wu et al., 2013b; Ji et al., 2013). In this work, these altered proteins confirmed the oxidative stress and subsequent cellular injury in cytoskeleton induced by metal pollution in oysters collected from BJ site.

Guanine nucleotide binding proteins, also known as G proteins, are involved in signaling pathways (Clapham and Neer, 1993). Recent studies demonstrated that G proteins may be critical for the stress responses, such as bacterial challenges (Clapham and Neer, 1993; Wu et al., 2013c). In marine mussel *Mytilus galloprovincialis*, one G protein was altered in response to bacterial challenges (Wu et al., 2013c). Cathepsin L played a major role in protein degradation of various physiological and

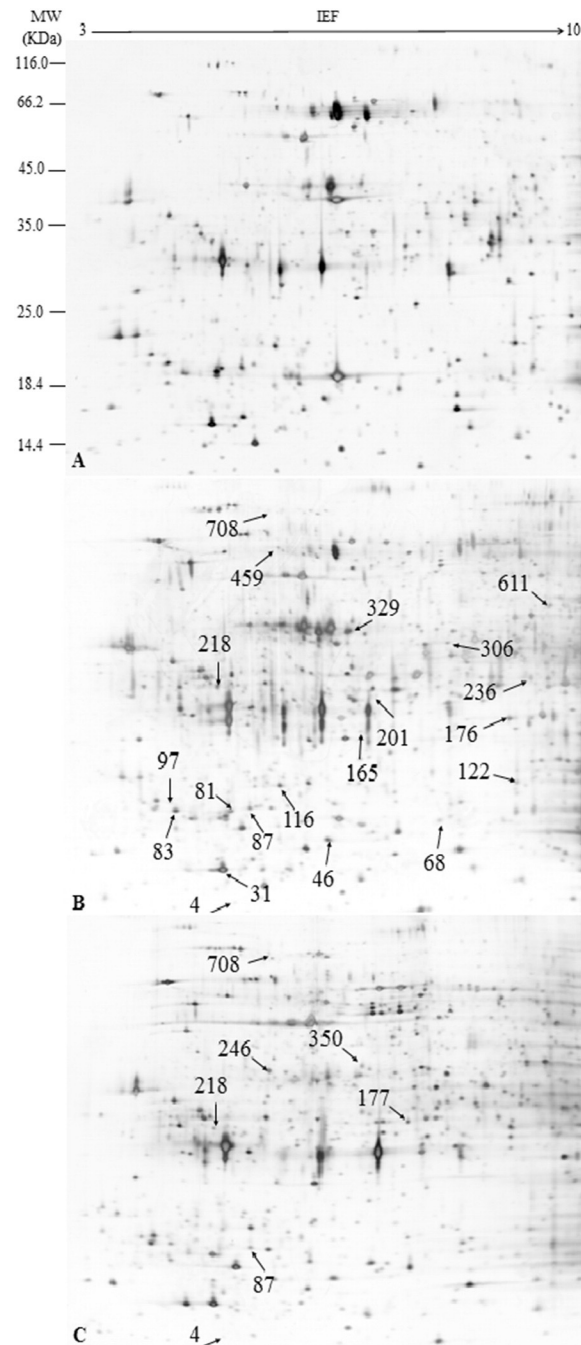


Fig. 3. Representative 2-DE images with dissolved protein spots from hepatopancreas tissues of oysters *Crassostrea hongkongensis* were submitted to isoelectric focusing on 4–7 IPG strips (24 cm) followed by electrophoresis on 12.5% SDS-PAGE. Gels were stained by silver stain. Gels (A, B and C) of oyster samples are from (A) JZ, (B) BJ and (C) FG sites, respectively. The protein spots observed in all three biological replicates were analyzed by MALDI-TOF/TOF mass spectrometry.

pathological processes and is a lysosomal cysteine protease involved in immune responses (Dorts et al., 2011). Ribosomal proteins perform the crucial function of protein biosynthesis and have been also recognized as immunogenic proteins (Ruszczuk et al., 2008). In bivalves and gastropods, the shell is mainly formed by CaCO₃ crystals. The calcium regulation is an important process for the shell formation, as well as other physiological processes including immune-defense mechanisms (Nikapitiya et al., 2010). Both EF-hand domain-containing protein and ependymin-related protein are involved in calcium homeostasis and have been found to be immune responsive in marine bivalves. The down-regulation of these two proteins indicated the immune stress induced by metal pollution in oysters from BJ site, combined the altered guanine nucleotide binding protein, cathepsin L and 40S ribosomal protein. In our previous study, cathepsin L was similarly down-regulated in the gills of clam *Ruditapes philippinarum* exposed to arsenate (Wu et al., 2013b). Interestingly, the arsenic concentration was significantly high in the hepatopancreas of oysters *C. hongkongensis* collected from BJ

site, which implied that cathepsin L might be the protein biomarker of arsenic pollution.

Adipophilin is an adipocyte differentiation-related protein and is proposed as a general marker for the lipid load of non-adipogenic cells (Heid et al., 1998). Fatty acid-binding protein in adipocyte is a small molecular-weight protein that has a high binding affinity for long-chain fatty acids. It is shown to coordinate the lipid responses in cells and plays an important role in fatty acid metabolism by transporting fatty acids from cell membrane to mitochondria for oxidation (Kleine et al., 1992). ATP synthase is responsible for the production of ATP for energy supply. Therefore the altered adipophilin, fatty acid-binding protein and ATP synthase implied the disturbance in lipid and energy metabolism induced by metal pollution in oysters from BJ site. Ferritin has two functions, iron detoxification and iron storage. This protein plays a vital role in the cellular homeostasis as the physiological source of iron for the cell by storing excess iron (Durand et al., 2004). In the oyster samples from BJ site, the very high concentration of Fe was observed (Table 1).

Table 2

List of protein spots that differentially expressed in oysters *Crassostrea hongkongensis* sampled from BJ and FG compared to those from JZ.

Spot ID ^c	Protein name	Accession number ^d	MW/Da	pI	Protein score ^e	SC (%) ^f	PN ^g	Fold changes ^h
Oxidation-reduction process								
4	SH3 domain-binding glutamic acid-rich-like protein 3	405952131	10,705	5.06	169	31	3	−7.46 ^a −7.48 ^b
81	Cytochrome b5	84619354	14,589	5.03	397	38	3	−1.71 ^a
Metabolism								
83	ATP synthase subunit delta, mitochondrial	405953462	18,396	5.19	38	5	1	−1.51 ^a
236	Cathepsin L	405958751	36,510	6.11	38	3	1	−1.85 ^a
459	Adipophilin	405954235	54,006	5.16	216	7	3	−1.77 ^a
Cytoskeleton								
41	Actin-depolymerizing factor 6	297347132	15,677	5.83	859	66	4	−1.95 ^a
87	Cofilin	405963691	18,605	4.92	147	9	1	−3.07 ^a −1.67 ^b
97	Myosin regulatory light chain sqh	405964694	19,552	4.68	152	24	3	−1.58 ^a
306	F-actin-capping protein subunit alpha	405951960	32,291	5.77	629	42	8	−1.54 ^a
350	Actin	2564711	41,765	5.3	331	20	6	1.67 ^a 1.54 ^b
611	Twinfilin-2	405968211	32,071	6.41	115	9	2	1.64 ^a
Protein biosynthesis								
46	40S ribosomal protein S12	405977575	14,900	5.79	369	32	3	−1.80 ^a
Chaperones								
68	Tubulin-specific chaperone A	405978204	12,774	5.71	298	30	2	−1.61 ^a
201	Alpha-crystallin B chain	405961891	23,113	5.74	280	21	3	−2.09 ^a
708	78 kDa glucose regulated protein	46359618	73,030	5.02	551	16	7	4.08 ^a 1.51 ^b
246	Heat shock protein beta-1	405961832	27,685	5.27	127	6	1	−1.70 ^b
116	Ferritin GF1	324792249	19,958	5.15	461	32	3	1.89 ^a
Ion homeostasis								
165	EF-hand domain-containing protein D2	405951006	23,011	5.3	73	7	2	−2.19 ^a
218	Mammalian ependymin-related protein 1	405968755	40,505	5.79	50	2	1	−1.56 ^a −1.62 ^b
Antioxidant enzymes								
122	Peroxiredoxin-5, mitochondrial	405974897	16,566	5.66	290	27	3	−1.92 ^a
176	Superoxide dismutase [Mn], mitochondrial	405962293	25,137	6.55	407	27	3	−1.54 ^a
Signal transduction								
329	Guanine nucleotide-binding protein subunit beta	405963261	37,306	5.62	307	14	4	3.05 ^a
Transport								
31	Fatty acid-binding protein, adipocyte	405949998	14,928	5.03	598	51	4	−2.68 ^a
177	Endoplasmic reticulum protein ERp29	405975720	28,273	5.19	29	4	1	2.13 ^b

^a Identification of differentially expressed proteins in oyster *Crassostrea hongkongensis* hepatopancreas from BJ and FG, compared to oyster hepatopancreas from JZ (less polluted).

^b Identification of differentially expressed proteins in oyster *Crassostrea hongkongensis* hepatopancreas from BJ and FG, compared to oyster hepatopancreas from JZ (less polluted).

^c Assigned spot ID as indicated in Fig. 3.

^d GI numbers in NCBI nr database.

^e Mascot score reported.

^f Sequence coverage.

^g Number of peptide sequences.

^h Fold changes with significant changes (>1.5 folds and $P < 0.05$) were calculated using ImageMaster 2D Platinum 7.0.

Therefore the ferritin GF1 was significantly ($P < 0.05$) up-regulated in the oyster samples from BJ site, which suggested that ferritin GF1 could be used as a biomarker of Fe contamination in oysters.

For the oyster samples from FG site, actin, cofilin and ependymin-related protein 1 were similarly altered compared with those in the oyster samples from BJ site. These altered proteins confirmed oxidative and immune stress induced by metal pollution in the oyster samples from FG site. However, two proteins including heat shock protein beta-1 and endoplasmic reticulum protein (ERp 29) were uniquely altered in the oyster samples from FG site. Cells respond to environmental stressors including physical (e.g. heat) or chemical (e.g. heavy metals) impacts by increased transcription of genes encoding so called heat shock or stress proteins. Heat shock proteins are ubiquitous molecular chaperones that are involved in the defensive system, such as anti-oxidative system, as mentioned above (Ji et al., 2013). ERp29 is a ubiquitously expressed endoplasmic reticulum (ER) stress-inducible protein that executes protective action by binding to denatured or aggregated cellular proteins thereby facilitating their refolding (Mkrtchian et al., 1998). These two altered proteins suggested the cellular injury induced by metal pollution in the oysters from FG site, via differential metabolic pathways compared with those in the oysters from BJ site.

The Jiulongjiang Estuary in South China has been severely polluted by metals, which posed great risk on the coastal organisms. As shown by the accumulations of metals/metalloid in oyster hepatopancreas, the two sampling sites, BJ and FG, were polluted by several metals, including Cr, Co, Ni, Cu, As, Ag, Cd and Pb. Especially, the oysters from BJ site were also contaminated by Fe and Zn. In this work, we applied two-dimensional electrophoresis (2-DE)-based proteomics to the oysters *Crassostrea hongkongensis* from metal pollution sites to characterize the proteomic responses induced by metal pollution. Proteomic responses indicated that the oysters from BJ site were more severely contaminated than those from FG site, which was consistent with the higher integrated metal contamination value of BJ site. Basically, metal pollution induced cellular injuries, oxidative and immune stresses in oyster hepatopancreas from both BJ and FG sites via differential metabolic pathways. In addition, metal pollution in BJ site induced disturbance in energy and lipid metabolisms in oysters. Results indicated that cathepsin L and ferritin GF1 might be used as the biomarkers of As and Fe in oysters *C. hongkongensis*, respectively. This study demonstrates that proteomics is useful to characterize the biological effects induced by metal pollution.

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