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An integrative biomarker approach to assess the environmental stress in the north coast of Shandong Peninsula using native oysters, *Crassostrea gigas*

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ABSTRACT

An integrative biomarker approach was employed to evaluate the environmental quality of the north coast of Shandong Peninsula along the southern Bohai Sea of China, where pollution is an imminent threat due to rapid urbanization and industrialization. A battery of biomarkers and the metal bioaccumulation in tissues of native oyster *Crassostrea gigas* were measured under field conditions. Integrative biomarker index (IBR) and metal body burden were calculated to differentiate the pollution status of seven sampling sites. According to our results, Xinzhuang (XZ) site was the most severely contaminated, with the highest IBR value of 3.58, while the lowest IBR value (0.04) was obtained at Penglai (PL). Such an integrated biomarker approach was proved as a useful method for environmental quality assessment in the study area.

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1. Introduction

The coastal and estuarine ecosystems in China are now severely influenced by pollution pressures. Various contaminants, especially elevated metal discharges from different sources, have entered the coastal and estuarine areas and caused degradation of aquatic environments (Wang et al., 2011). Bohai Sea is a nearly enclosed interior sea of northeast China (Meng et al., 2008). Previous studies have recorded that Bohai Sea and the nearby coastal areas and estuaries were suffering severe problem of metal pollution (Wang et al., 2011; Xu et al., 2013). The north coast of the Shandong Peninsula, located along the southern Bohai Sea, is one of the most important economic zones and industrial clusters in Northeast China. Heavy metals distribution and contamination in the surface seawater and sediment (Xu et al., 2013; Gao et al., 2014) as well as bivalve species (Liang et al., 2004; Wang et al., 2005; Li and Gao, 2014) have been documented in this region. However, there is little information about biological effects-based monitoring of such metal contamination in this region.

Presently, a wide range of biomarkers has been increasingly used as sensitive early warning tools for the assessment of environmental variables caused by chemical contaminants in coastal areas (Campillo et al., 2013; Marigomez et al., 2013a; Bellas et al., 2014). Oxidative stress is essentially an imbalance between cellular production of reactive oxygen species (ROS) and an organism's ability to detoxify the reactive intermediates. This is an increase in evidence revealing that exposure to contaminants could induce the production of ROS, resulting in toxic effects on antioxidant system as well as cellular damage, protein degradation and DNA damage (Livingstone et al., 1993). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) have been frequently used as effect biomarkers to assess the contaminated area by caged and native bivalves (Leinio and Lehtonen, 2005; Viarengo et al., 2007; Campillo et al., 2013; Turja et al., 2013). Glutathione S-transferases (GSTs) represent a main group of phase II detoxification enzymes involved in the detoxification of organic pollutants (Sheehan et al., 2001). Additionally, lysosomal membrane stability (LMS) is also considered as an effect biomarker of cell well-being (Aguirre-Martinez et al., 2013), and the measurement of LMS has been well-established as a useful tool to investigate the general stress of large range of organisms (Moore et al., 2004; Broeg et al., 2005). Comet assay has been extensively used as a rapid and sensitive tool to evaluate genotoxicity effect in aquatic organisms (Mitchelmore and Chipman, 1998; Valavanidis et al., 2006). Furthermore, stress-on-stress (SOS) response, which is calculated as the survival time in air, was also used as an indicator of general health of organisms impacted by environmental stressors (Viarengo et al., 2007). Moreover, acetylcholinesterase (AChE) has been widely used as enzymatic biomarker of neurotoxicity for aquatic organisms after exposure to pollutants

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(Rickwood and Galloway, 2004). Numerous previous studies have also demonstrated metallothionein (MT) as an indicator of heavy-metal exposure (Viarengo et al., 1997; Zorita et al., 2005).

Bivalve species have been widely used as bio-indicator for risk assessment of chemical pollution in coastal and estuarine area, due to their sessile life style, wide geographical distribution and ability to accumulate contaminants from the surrounding water (O'Connor, 2002; Rainbow, 2002). The aim of this study was to investigate the biological effects of native bivalves at different sampling sites along north coast of the Shandong Peninsula using a multi-biomarker approach. In the present study, the Pacific oyster (*Crassostrea gigas*) was selected because of its wide distribution in coastal and estuarine area (Jeng et al., 2000; Yu et al., 2013). To follow the integrated assessment approach, a battery of effect biomarkers including SOS, SOD, CAT, LPO, GST, LMS and DNA damage (COMET), and exposure biomarkers including AChE and MT were employed to assess the risk of environmental contamination in this coastal area.

2. Materials and methods

2.1. Site selection

Seven sites were selected along north coast of the Shandong Peninsula (Fig. 1), including Yangkou (YK), Xinzhuang (XZ), Penglai (PL), Zhifudao (ZFD), Xin'an (XA), Muping (MP), and XiaoShidao (XSD). The sites were selected according to their potential environmental pressure and historical data (Wang et al., 2005; Xu et al., 2013). YK, XZ, PL, XA and MP are estuarine areas, while ZFD and XSD are traditional aquaculture areas.

2.2. Collection and sample processing

Pacific oyster *C. gigas* (4–8 cm shell length) was collected randomly from each site for biological and chemical analysis in October 2014. Sample preparation was carried out in the field immediately to avoid the disturbance of transportation or confounding factors (Chandurvelan et al., 2013a), as recommended by Marigomez et al. (2013a). Oysters used for chemical analysis and SOS response were put into clean polypropylene container, and transferred refrigerated to the laboratory. Animals used for biometric analysis and immune responses were put into aerated tanks filled with seawater from the sampling sites. In order to minimize the changes in physiological processes such as digestion and redox status during transportation, the digestive glands and gills of twenty-two individuals (12 for the determination of oxidative stress, AChE activity and MT content; 10 for additional analysis if required) from each site were sacrificed, then transported in dry ice to the laboratory and stored at −80 °C. The physicochemical quality of seawater, temperature (degrees Celsius), salinity (psu), dissolved oxygen (milligrams per liter) and pH were determined at each sampling site using an YSI multi-parameter probe (YSI Incorporated, Yellow Springs, OH, USA) (Table. 1).

2.3. Metal determinations

Oyster samples (n = 20) from each site were divided into four pools, dissected and dried at 60 °C to constant weight. Approximately 0.05–0.1 g dry tissues were digested with concentrated nitric acid at 120 °C for 2 h, then diluted with 0.1 M nitric acid and analyzed by ICP-MS (PerkinElmer, Elan DRC II, USA). Merck standard reference solutions for calibration were diluted in 0.1 M nitric acid. Eight metals, including Pb, Co, Ni, As, Cd, Zn, Fe and Cu, were analyzed. The metal concentrations in tissues are shown as μg metal g^{-1} tissue dry-wt.

2.4. Effect biomarkers

2.4.1. "Stress on stress" response

The survival in air (SOS response) was tested as described by Viarengo et al. (1995). 30 oysters from each site were placed in a plastic box, and exposed under controlled humidity chambers at 18 ± 1 °C. Mortality was recorded daily, and the mortality curve was estimated. The median survival time (LT_{50}) was calculated for the time at which 50% of individuals died.

2.4.2. Oxidative stress biomarkers

Tissues of digestive glands and gills from oysters (n = 12) of each site were pooled into 6 replicates. Samples were homogenized in 1:5 (w:v) ice-cold phosphate buffer (containing 100 mM KH_2PO_4/K_2HPO_4,

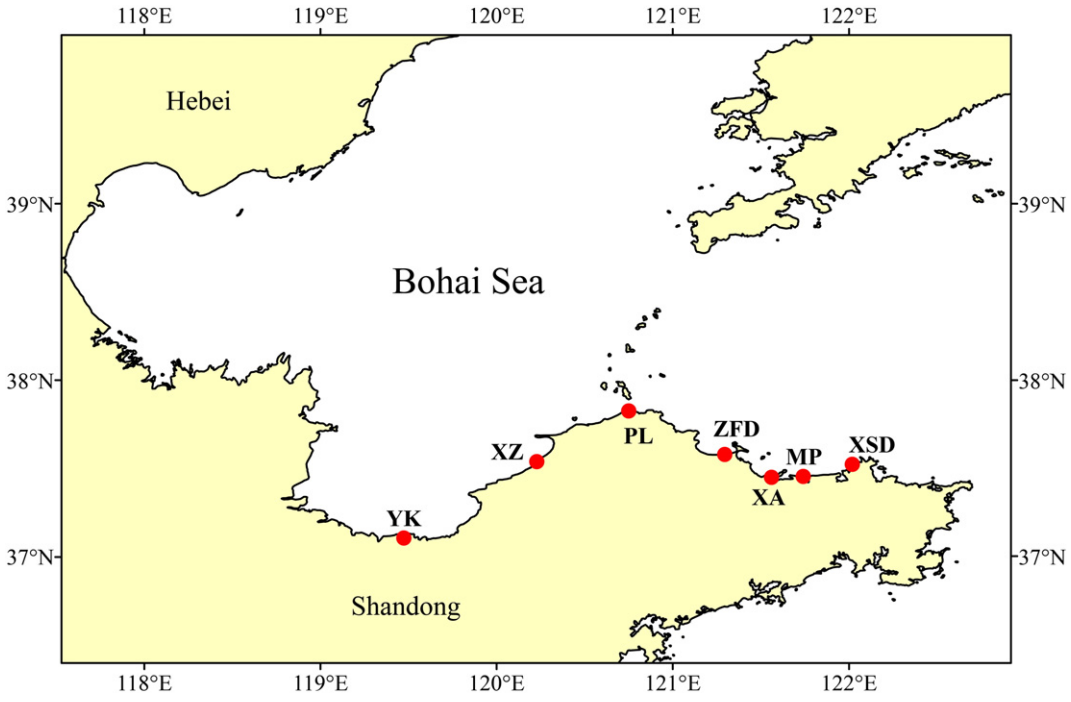

Fig. 1. Map showing the oysters sampling sites.

Table. 1
Physical and chemical parameters (*T*, temperature; Sal, salinity; DO, dissolved oxygen; and pH) at seven sampling sites.

Parameters	Site						
	YK	XZ	PL	ZFD	XA	MP	XSD
T (°C)	19.4	19.6	18.7	19.0	19.0	18.8	19.0
Sal (psu)	30.3	31.5	33.0	29.5	31.3	32.0	32.0
DO (mg/L)	6.3	6.9	6.3	7.3	7.5	8.5	7.2
pH	7.8	8.0	7.8	8.3	8.1	8.4	8.0

Values represent the mean of temperature, salinity, dissolved oxygen and pH.

1 Mm EDTA, pH 7.4), and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatants were used for the measurement of SOD, CAT activities and the MDA content with a microplate spectrophotometer (Infinite M200, TECAN, Switzerland). The protein concentration in the supernatant was measured according to the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

SOD activity was measured using the method described by Flohe and Otting (1984). One unit of SOD activity (U) was defined as 50% inhibition of the nitobluetetrazolium chloride (NBT) photoreduction rate and expressed in U (mg protein)$^{-1}$. CAT activity was measured using the method described by Buege et al. (1977). One unit of CAT activity (U) was defined as micromoles of hydrogen peroxide (H_2O_2) degraded per min per milligram of protein and express in U (mg protein)$^{-1}$. Lipid peroxidation (LPO) level was assayed by measurement of malondialdehyde (MDA) contents, according to Buege et al. (1977). The reaction was determined at 532 nm using thiobarbituric acid (TBA) reagent, and MDA content wad defined as the amount of thiobarbituric acid reactive substance (TBARS). Results were expressed as nmol MDA (mg protein)$^{-1}$.

2.4.3. Phase II detoxification enzyme

Glutathione S-transferase (GST) activity was measured using 1-chloro-2,4-dinitraobenzene (CDNB) as substrate, according to Habig et al. (1974). One unit of GST was expressed as the formation of nmol CDNB conjugates min^{-1} (mg protein)$^{-1}$ using a molar extinction coefficient ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

2.4.4. Lysosomal membrane stability

LMS test was determined by the neutral red retention (NRR) assay following the methodology proposed by Lowe et al. (1995) with some modifications described by Aguirre-Martinez et al. (2013). Briefly, 40 μL of haemocytes suspension was spread on microscope slides, transferred to a lightproof humidity chamber and allowed to attach. After incubation for 30 min, 40 μL of 0.2 mM neutral red solution was added to start the reaction. The haemocytes were observed under microscope after 15, 30, 60 and 90 min, and the NRR time was defined as the time when 50% the cells display spillage from the lysosomes into the cytosol (Martinez-Gomez et al., 2008).

2.4.5. Alkaline single cell gel electrophoresis (comet assay)

The comet assay was performed according to a modified method of Danellakis et al. (2011), following the protocol proposed by Olive et al. (1991). Briefly, 40 μL of haemocytes suspension was mixed with 70 μL of 1.0% low melting point agarose, gently pipetted onto the glass slides pre-coated with 2.0% normal melting point agarose, and then covered with a coverslip. After solidification, the slides were placed in chilled lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% (v/v) Triton X-100 and 10% (v/v) DMSO, pH 10.0) in the dark for 1 h. Slides were placed in an gel electrophoresis tank, and covered with freshly prepared alkaline electrophoresis solution (75 mM NaOH, 1 mM EDTA, pH > 12.0) for 20 min at 4 °C to allow DNA unwinding. After electrophoresis (25 V, 300 mA for 10 min), slides were placed into a staining tank and neutralize by Tris buffer (0.4 M Tris-HCl, pH 7.4). DNA was stained with SYBR Green I and examined using a fluorescence microscope. Results were expressed as the percentage of DNA in comet tail (% DNA in tail).

2.5. Exposure biomarkers

2.5.1. AChE activity assay

The activity of AChE was determined in gills using the colorimetric method described by Ellman et al. (1961). Samples were homogenized in 1:2 (w:v) Tris-HCl buffer (0.1 M Tris-HCl containing 0.1% Triton X-100, pH 7.0), and the homogenates were centrifuged at 10,000*g* for 20 min at 4 °C. One unit of AChE activity was expressed in nmol (min mg protein)$^{-1}$ using a molar extinction coefficient ($\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$).

2.5.2. Total metallothionein content

Metallothionein (MT) content was measured following the spectrophotomertric method proposed by Viarengo et al. (1997), with some modifications described by Chandurvelan et al. (2013b). Briefly, tissues were homogenized in 1:3 (w:v) volumes of homogenizing buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, with added 0.006 mM leupeptine, 0.5 mM PMSF and 0.01% β-mercaptoethanol, pH 8.6) and centrifuged at 10,000*g* for 45 min at 4 °C, diluted supernatants were pipetted into centrifuge tubes, then cold absolute ethanol and chloroform were added to pure the metalloprotein. After centrifuged at 6000*g* for 10 min at 4 °C, the supernatant was collected and added to cold ethanol. The samples were incubated at 20 °C for 1 h before centrifugation at 6000*g* for 10 min at 4 °C. The resultant pellet was re-suspended in Tris-HCl buffer, then added Ellman's reagent (0.43 mM DTNB in 200 mM KH_2PO_4, pH 8.0), and centrifuged at 3000*g* for 10 min at 4 °C. Reduced GSH standard solutions were used for calibration. The absorbance of supernatant was measured at 412 nm, and the results were expressed as μg MT (mg protein)$^{-1}$.

2.6. Integrative biomarker indices

Six biomarkers (COMET, LMS, MDA, CAT, SOD and SOS) were integrated in the IBR index (Beliaeff and Burgeot, 2002). Since the IBR value is directly dependent on the number of biomarkers in the set, the obtained IBR value must be divided by the number ($N = 6$) of biomarkers used and termed as IBR/n (Broeg and Lehtonen, 2006). Biomarker were ordered according to their level of biological complexity from genotoxicity (COMET) to population fitness (SOS), with subcellular (LMS), cellular (MDA), and tissue (CAT, SOD) biomarkers in between.

The IBR was calculated by summing up triangular star plot areas calculated for each two neighboring biomarkers in a given data set. The following procedures were adopted: (1) calculation of mean and standard deviation for each biomarker of the sampling stations, (2) standardization of data for each station: $Y = (X - m) / s$, where Y = standardized value of the biomarker, X = value of each biomarker response, m = mean value of the biomarker, and s = standard deviation of the biomarker, and (3) the score (S) was computed as: $S = Y + |\min|$, where $S \geq 0$ and $|\min|$ = absolute minimum value of Y for each biomarker. The score of biomarker was visualized using a star plot in which the radial coordinate corresponded to the score. When S_i and S_{i+1} were assigned as two consecutive clockwise scores of a given star plot, the area of the star plot (IBR value) obtained from the sum of the six triangular areas was calculated as follows: $IBR = \mathrm{Sin}\alpha\ [(S_i \times S_{i+1}) / 2 + (S_{i+1} \times S_{i+2}) / 2 + \ldots + (S_{n-1} \times S_n) / 2]$, where α = angle (in radians) formed by each two consecutive axis, and n = the number of biomarkers.

2.7. Statistical analysis

The experimental data were expressed as mean ± standard deviation. Raw data were analyzed for normality and variance homogeneity

Table 2
Concentrations of metals (μg g^{-1} dry weight) measured in the whole tissue of oysters collected from seven sampling sites.

	YK	XZ	PL	ZFD	XA	MP	XSD
Pb	0.06 ± 0.01^{a}	0.62 ± 0.03^{c}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.08 ± 0.02^{b}	0.05 ± 0.01^{a}	0.04 ± 0.00^{a}
Co	0.02 ± 0.00^{a}	0.14 ± 0.01^{d}	0.02 ± 0.00^{a}	0.03 ± 0.01^{b}	0.05 ± 0.01^{c}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}
Ni	0.08 ± 0.02^{a}	0.31 ± 0.08^{c}	0.10 ± 0.02^{a}	0.10 ± 0.04^{a}	0.18 ± 0.02^{b}	0.08 ± 0.01^{a}	0.06 ± 0.02^{a}
As	0.64 ± 0.04^{a}	5.00 ± 0.34^{d}	1.00 ± 0.12^{b}	0.74 ± 0.14^{a}	1.40 ± 0.09^{c}	0.80 ± 0.16^{ab}	0.61 ± 0.05^{a}
Cd	0.39 ± 0.04^{a}	5.69 ± 0.24^{c}	0.42 ± 0.07^{a}	0.69 ± 0.09^{b}	0.31 ± 0.02^{a}	0.32 ± 0.02^{a}	0.71 ± 0.08^{b}
Zn	71.44 ± 14.41^{a}	1329.76 ± 166.78^{b}	84.93 ± 13.23^{a}	95.81 ± 4.21^{a}	104.15 ± 16.95^{a}	61.62 ± 10.10^{a}	29.93 ± 1.88^{a}
Fe	18.15 ± 1.01^{ab}	62.92 ± 8.57^{d}	13.62 ± 2.48^{ab}	20.49 ± 4.74^{b}	35.10 ± 9.97^{c}	15.59 ± 4.41^{ab}	10.42 ± 2.31^{a}
Cu	13.16 ± 2.87^{a}	585.53 ± 99.07^{b}	15.76 ± 2.56^{a}	18.45 ± 0.91^{a}	22.92 ± 2.49^{a}	9.46 ± 2.27^{a}	10.78 ± 1.04^{a}

Different letters (a, b, c, d) denote statistically significant differences between pairs of means according to the Duncan's test, $P < 0.05$.

using the Shapiro-Wilk and Levene's test, respectively. One-way analysis of variance (ANOVA) and Duncan's test were applied to determine significant differences ($P < 0.05$) in various biomarkers between different sites. All analyses were carried out with the SPSS 16.0 software (SPSS Inc., Chicago, Illinois). Principal component analysis (PCA) was accomplished using MATLAB.7.0 statistical software (The MathsWorks Inc., Natick, USA) to assess the variability associated with biological biomarkers (AChE, MT, SOD, GST, MDA and CAT), using data from the same individual at each sampling site.

3. Results

3.1. Concentrations of metals in tissues

The metal concentrations (Pb, Co, Ni, As, Cd, Zn, Fe, Cu) in soft tissues of *C. gigas* are shown in Table 2. The highest concentrations of all analyzed metals were recorded at XZ site with the concentration of Pb, Co, Ni, As, Cd, Zn, Fe and Cu reaching 0.62, 0.14, 0.31, 5.00, 5.69, 1329.76, 62.92 and 585.53 μg g^{-1} dry weights of tissues, respectively. Oysters from XSD site showed the lowest metal concentrations.

3.2. Effect biomarkers

The LT_{50}values and survival curves of air exposure for all sampling sites are shown in Fig. 2A and Fig. 2B, respectively. It was found that all the LT_{50} values ranged from 6 to 11 d. Oysters sampled from XZ (LT_{50} = 11 d) appeared to be more resistant, whereas oysters from XA, MP and YK were less resistant, and the oysters from XSD (LT_{50} = 6 d) displayed the least resistance to air exposure.

In the digestive glands, significant differences in the biomarkers of oxidative stress were observed among all sampling sites. The highest SOD activity was detected at XZ site (29.12 ± 4.41 U mg^{-1} protein^{-1}), and the lowest was found at MP site (Fig. 3A). In contrast, the lowest CAT activity was recorded at XZ site (24.99 ± 4.48 U mg^{-1} protein^{-1}), while the highest was recorded at PL and XSD sites (Fig. 3B). Variation in MDA content was also observed at different sampling sites with the lowest MDA level recorded at PL and YK and the highest level at XZ site (Fig. 3C). In addition, oysters from XZ presented a significantly higher GST activity than other sampling sites (Fig. 3D).

Neutral red retention (NRR) assay was used to evaluate lysosomal membrane integrity in haemocytes of oysters collected from different sampling sites. A significant higher NRR time was recorded in the haemocytes of oysters from PL site (81 min) (Fig. 4A), while oysters at XZ site had the lowest NRR time (30 min). There were no significant differences in NRR time at sites of XA, ZFD, MP, XSD and YK.

In addition, the haemocytes of oysters sampled from XZ and MP exhibited the highest DNA damage values, reaching 18.15% and 13.87%, respectively (Fig. 4B). However, no significant difference was observed in the degree of DNA damage among other sampling sites.

3.3. Exposure biomarkers

There was no significant difference in AChE activity of oysters from XA, ZFD, PL, XSD and YK sites (Fig. 4C), and the highest (1.66 ± 0.17 nmol mg^{-1} min^{-1} protein^{-1}) and lowest AChE activity (0.57 ± 0.29 nmol mg^{-1} min^{-1} protein^{-1}) was observed at MP and XZ sites, respectively.

The MT content in oysters' digestive glands demonstrated significant variations at different sampling sites (Fig. 4D). The highest accumulation of MT (238.24 ± 10.82 μg MT mg^{-1} protein^{-1}) was found in oysters sampled from XZ site, whereas the lowest MT concentration was recorded at PL and XSD sites. However, there was no significant difference at sites of YK, MP and ZFD.

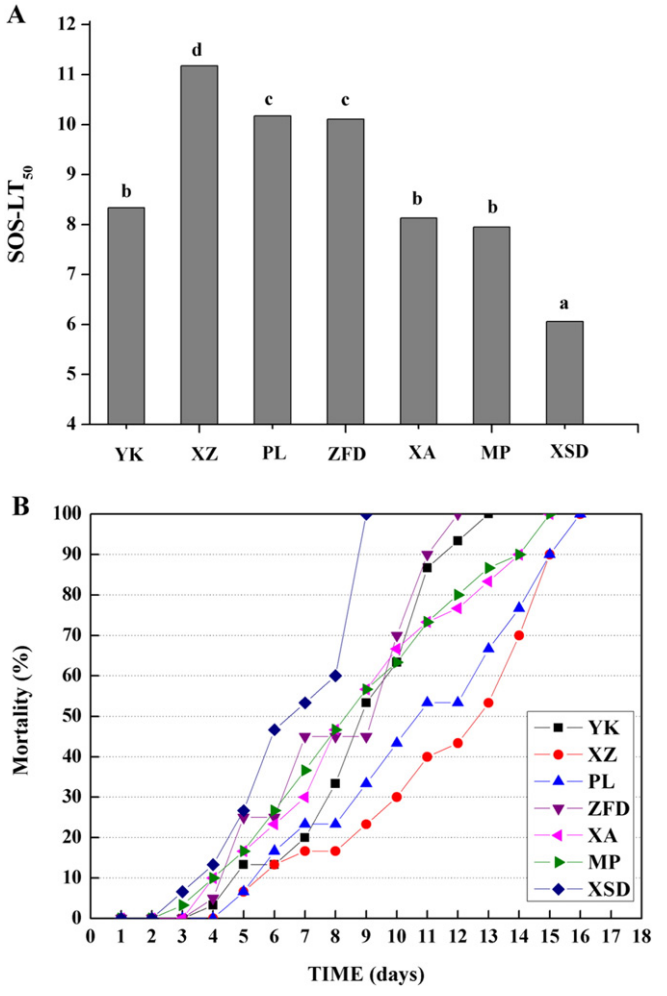

Fig. 2. Population fitness biomarker: (A) SOS response: time to kill 50% of individuals collected from each station; (B) Accumulated mortality in oysters against air exposure time (days). Different letters (a, b, c, d) represent significant differences (Duncan's test, $P < 0.05$).

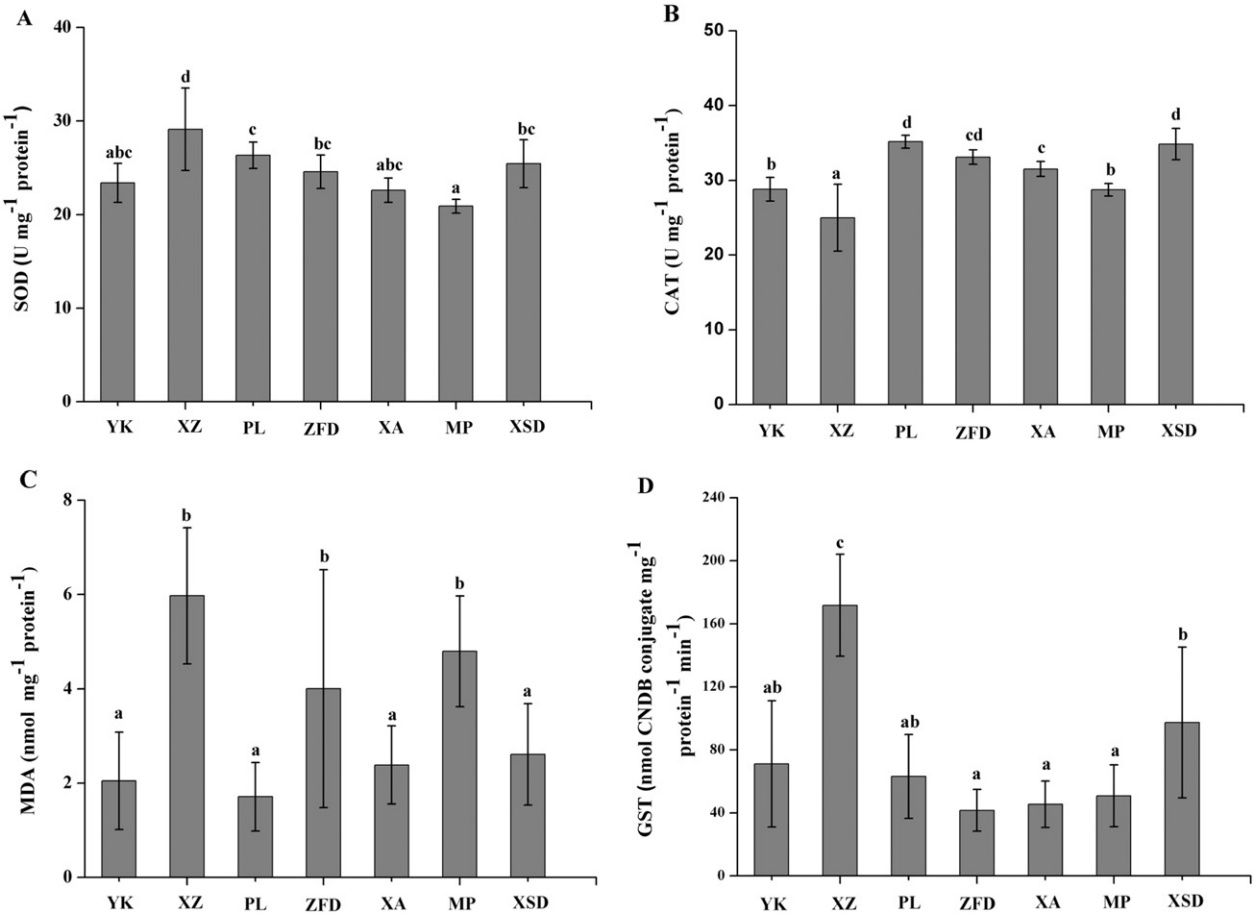

Fig. 3. Antioxidant enzyme biomarkers in digestive glands of *Crassostrea gigas*: (A) SOD activity; (B) CAT activity; (C) MDA content; (D) GST activity. Each bar represents the mean value from six determinations with standard deviation, and bars with different letters are significantly different (Duncan's test, $P < 0.05$).

3.4. Principal component analysis

Two main factors explaining 69.63% of the total data variance were extracted from the results of PCA. The first component (PC 1) accounted for 49.93% of the total variance, and the results show that oysters sampled from XZ were clearly different from other sites by its location on the positive side of PC 1 (Fig. 5). The high levels of SOD, MDA, GST, MT and low levels of AChE and CAT defined this separation. On the contrary, oysters from YK, PL, XA, MP and XSD were on the negative side of PC1 due to their high levels of CAT and AChE. The second component (PC 2) explained 19.70% of the total variance. The oysters from PL and XSD were located on the positive side of PC 2, which was characterized by high CAT loading. On the contrary, the oysters collected from XA, YK and MP were on the negative side of PC 2. In addition, there was no significant biomarker response which could be identified from oysters collected at ZFD site.

3.5. Integrated biomarker indices

The IBR star plots in Fig. 6 present the response of the six biomarkers (COMET, LMS, MDA, CAT, SOD and SOS) and Fig. 7 shows the whole biomarker IBR/n values for each sampling site. The lowest IBR/n value was found in oysters sampled from PL (IBR/n = 0.04) whereas the highest IBR/n value was detected at XZ (IBR/n = 3.58) site. Moderate IBR/n values were found at MP (IBR/n = 0.76) and ZFD (IBR/n = 0.49), followed by IBR/n values of 0.42, 0.24 and 0.12 at YK, XA and XSD sites, respectively.

4. Discussion

During the last decade, several studies have investigated metal contaminants in surface water, sediment, as well as aquatic organisms along the north coast of the Shandong Peninsula (Liang et al., 2004; Wang et al., 2005; Xu et al., 2013; Gao et al., 2014; Li and Gao, 2014). In the present study, chemical analysis of oyster tissues was performed to identify contaminant conditions, and several physiological parameters and biomarkers indicative of oxidative stress, neurotoxicity and genotoxicity were also investigated to assess the health status of selected organisms.

Oysters sampled from XZ site showed significantly higher metal concentrations than that collected from other sites. The relatively high levels of Zn, Cu and Fe within the soft tissues can be attributed to the greater metal bioavailability than other trace metals. Similar bioaccumulation pattern was also reported in other aquatic organisms. For example, some fish and shellfish accumulated more Zn, Cu and Fe than non-essential metals such as lead, mercury and cadmium (Anan et al., 2005; Liu et al., 2014; Paez-Osuna and Osuna-Martinez, 2015). The concentration of Cd, Pb and As from each sampling site had a wide interval ranging from 0.31 (YK) to 5.69 μg g^{-1} (XZ), 0.04 (XSD) to 0.62 μg g^{-1} (XZ), 0.61 (XSD) to 5.00 (XZ) μg g^{-1}, respectively. The Cd levels in the *C. gigas* from XA, ZFD, PL, MP, XSD and YK sites were all below the safety guideline concentration (2.00 μg g^{-1} wet weight) of AQSIQ (2001), while the As levels from XZ, PL and YK were higher than the safety level of 1.00 μg g^{-1} wet weight according to the WHO (1982). In addition, the Pb level in the oyster tissues from all the sampling sites was below the guideline concentration (1.50 μg g^{-1} wet weight) from the European Commission (2006).

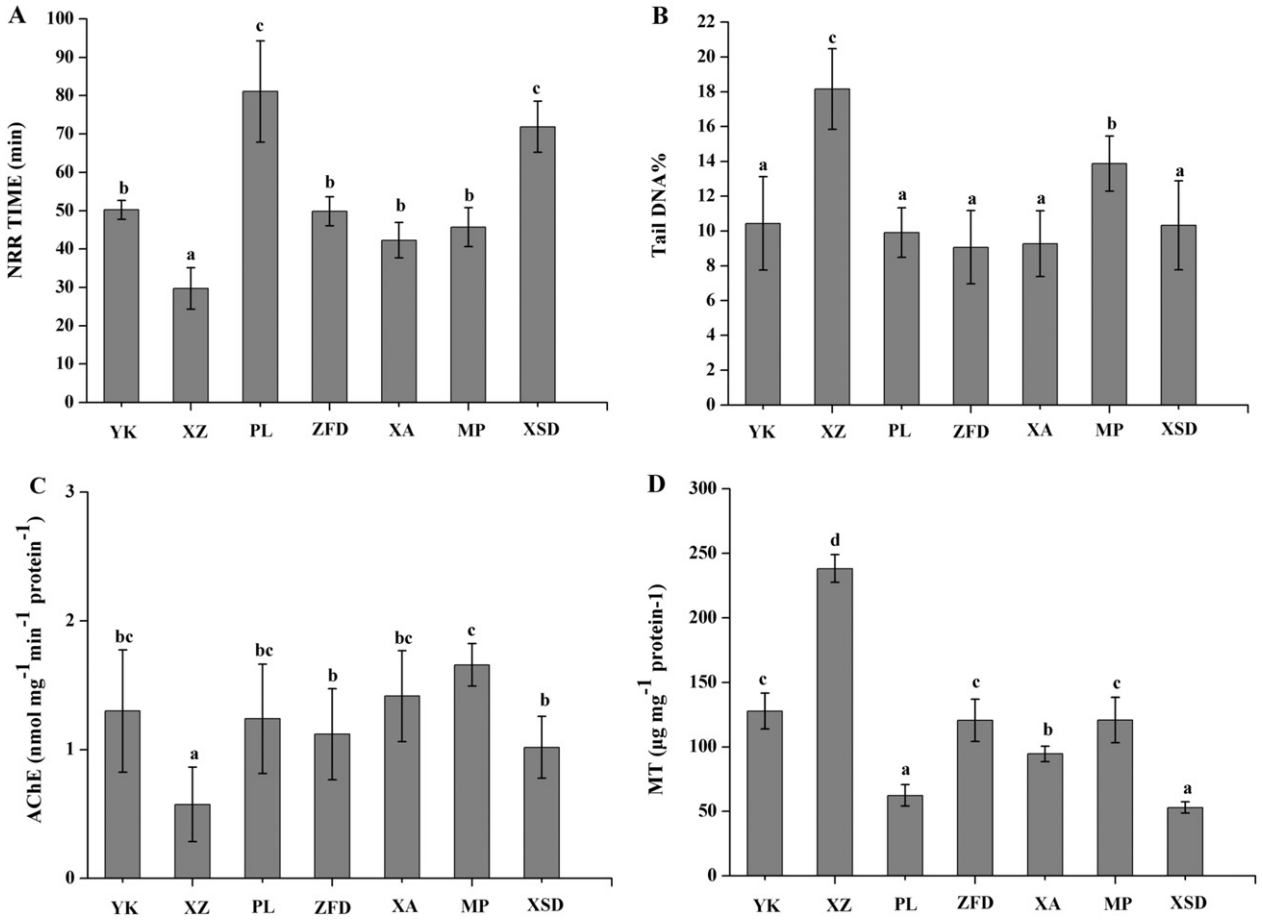

Fig. 4. Biomarkers: (A) Lysosomal membrane stability in haemocytes; (B) DNA damage in haemocytes; (C) AChE activity in gills; (D) MT content in the digestive glands. Each bar represents the mean value from six determinations with standard deviation, and bars with different letters are significantly different (Duncan's test, $P < 0.05$).

The measurement of survival in air (SOS) response is a physiological biomarker used to evaluate bivalve resistance to air exposure, which provides a simple and sensitive indicator of environment health (Viarengo et al., 2007). Many studies have demonstrated that exposure to contaminants could reduce the tolerance of bivalves to anoxia (de los Rios et al., 2013). However, the oysters collected at XZ site (with the highest levels of metals) were more resistant to anoxia in the present study. It was postulated that highly contaminated stress might strengthen the tolerance of the animals to adverse environment. Similarly, native oysters sampled from highly contaminated sites exhibited more tolerance than those from less contaminated sites (Hellou and Law, 2003).

Oxidative stress is a result of an increased accumulation of oxyradicals and ROS (Leonard et al., 2004; Weissenberg et al., 2010). Various environmental stresses lead to excessive production of ROS,

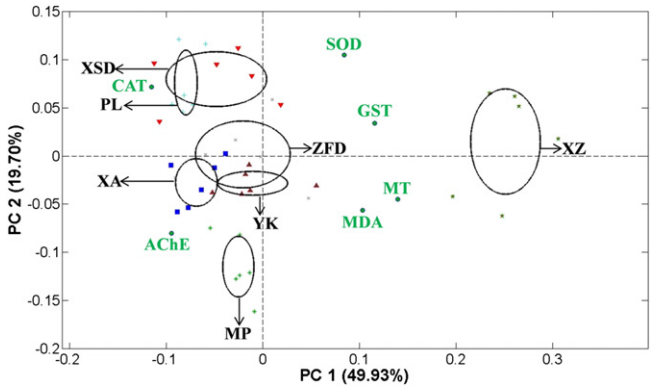

Fig. 5. The biplot containing PC scores of oyster tissues from seven sampling sites. XSD (▼), MP (*), XA (■), PL (+), ZFD (×), YK (▲) and XZ (★) and variables (six biological indices: SOD, CAT, GST, AChE, MDA and MT) contributions for the clustering of oyster samples. Ellipses represent mean ± standard deviation for each group of samples from different sites.

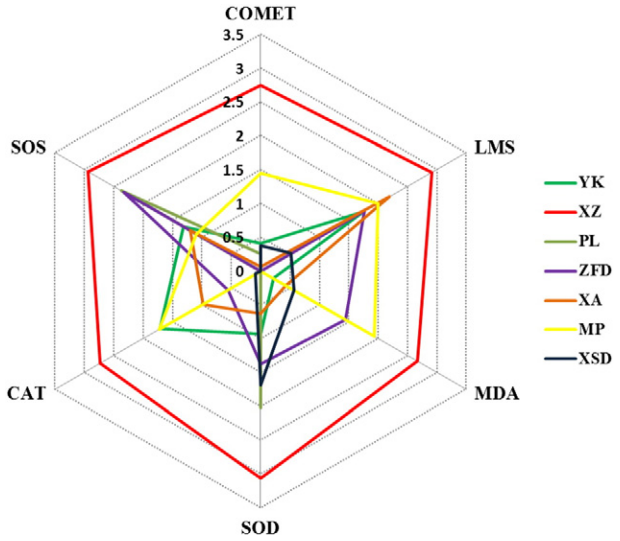

Fig. 6. IBR star plots of the biomarkers in *Crassostrea gigas* collected from seven sampling sites. COMET = DNA damage; LMS = lysosomal membrane stability; MDA = lipid peroxidation; SOD = superoxide dismutase; CAT = catalase; SOS = stress-on-stress response.

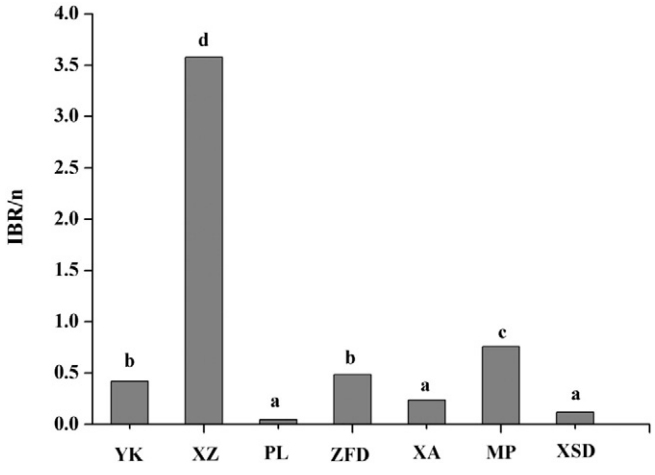

Fig. 7. Integrative biomarker response index (IBR/n) calculated using the biomarkers assessed in the present study (COMET, LMS, MDA, SOD, CAT and SOS; n = 6).

which can cause progressive oxidative damage and ultimately cell death (Galaris and Evangelou, 2002; Shi et al., 2004). In the present study, significant variations of SOD, CAT activities and MDA content were recorded in the digestive glands of oysters from each sampling site. Both SOD and CAT are primary defense enzymes involved in the anti-oxidative system (Giarratano et al., 2014). SOD catalyzes the conversion of superoxide anion to hydrogen peroxide and oxygen (Wang et al., 2014), while CAT converts the hydrogen peroxide to H_2O and molecular oxygen. In this study, higher SOD activity in the digestive glands of oysters from XZ site indicated a generally increased anti-oxidative response in the relatively polluted area. However, the CAT activity of oysters from XZ was significantly inhibited compared to other sites, which was likely related to higher levels of heavy metals at this site. Previous studies have recorded that mussels caged in the polluted sites showed lower CAT activity than that in the reference mussels (Viarengo et al., 2007; Campillo et al., 2013). On the other hand, the oxidative stress in organisms may also induce lipid peroxidation, resulting in increased MDA level (Kamel et al., 2014). In the present study, significantly higher MDA levels were found at XZ, MP and ZFD than other sites. The increased MDA level at XZ site was probably caused by bioaccumulation of heavy metals at higher level. However, the high MDA levels at MP and ZFD suggested that other potential pollutants might affect the biomarkers of oxidative stress. GST also plays an important role in anti-oxidative defense system, which is involved in the phase II biotransformation process and cellular detoxification of xenobiotic compounds (Regoli et al., 2002). In the present study, the highest GST activity was found at XZ site, which could be correlated to the highest concentrations of metals at this site. The high GST activity in the digestive glands may be able to compensate the low CAT activity in the tissues, since GST is also involved in the peroxidase activity (Barata et al., 2005).

AChE is a key enzyme commonly inhibited by xenobiotic compounds such as organophosphate, carbamate pesticides, polycyclic aromatic hydrocarbon, metals and surfactants (Lionetto et al., 2003; Sole et al., 2010; Gonzalez-Rey and Bebianno, 2014). In the present study, significant inhibition of AChE activity was observed in oysters from XZ, which was coincided with the elevated metal concentrations. Similar results were also observed in caged mussels and clams from heavily polluted areas (Barhoumi et al., 2014; Tsangaris et al., 2014), and native oysters from Pacific estuary where they were impacted by pesticides and heavy metals (Bernal-Hernandez et al., 2010).

MT was involved in various physiological processes such as homeostasis, metabolic regulation, protection against metals, oxidant damage as well as redox control in aquatic invertebrates (Mao et al., 2012). In this study, MT content in oysters from XZ was significantly higher than that from other sites. Several studies have found that MT content had positive correlations with metal concentrations in bivalves (Trombini et al., 2010; Khati et al., 2012). Furthermore, Cd, Cu and Zn were also regarded as an inducer of MT (Viarengo et al., 1997; Marigomez et al., 2002), which was consistent with the high levels of Cd, Cu and Zn at XZ site.

LMS is directly related to immune-reactivity in bivalves since lysosomes play a central role in the degradation of phagocytized materials, and thus lysosomes' alterations may result in immunity impairment (Martinez-Gomez et al., 2008). Previous studies suggested that LMS in mussel haemocytes constitutes a very useful indicator of cellular damage (Aguirre-Martinez et al., 2013; Lekube et al., 2014). In the present study, the NRR value of native oysters from different sites ranged from 30 to 81 min with the lowest NRR recorded at XZ site. These results were in agreement with previous studies that reduced NRR was detected in mussels and clams following exposure to contaminants such as metals, organic xenobiotics and antibiotic drugs (Lowe and Moore, 1979; Dailianis et al., 2003; Nigro et al., 2006; Rank et al., 2007; Aguirre-Martinez et al., 2013). DNA damage can lead to reproductive impairment, abnormal development and lethal mutations, which has been used as a general stress biomarker to indicate the genotoxic risk (Anguiano et al., 2007; Barranger et al., 2014; Vazquez-Boucard et al., 2014). The present study clearly demonstrated that oysters from XZ and MP were suffered from significant DNA damage.

Principal component analysis was performed to summarize the correlation between biomarker responses and study sites, and identify which groups of variables were responsible for this discrimination. In the present study, XZ site showed significantly positive correlation to the majority of biomarkers including SOD, MDA, GST and MT according to PC 1, which was likely related to the higher concentrations of metals measured at this site. Besides, high level of CAT was observed at PL and XSD sites according to PC 2, which was in agreement with the low metal concentrations at these sites. On the contrary, oysters from XA and MP were clearly differentiated from other sites by their higher AChE activity. The present findings suggested that these four sites (PL, XSD, XA, MP) were less impacted by metal contamination. In addition, YK and ZFD sites were suffering moderate levels of biomarker response in oyster tissues compared to that from other sites.

The IBR index is a powerful tool to assess the sensitivity of organisms to contaminants (Beliaeff and Burgeot, 2002; Serafim et al., 2012), which may be used to distinguish between polluted and less polluted areas (Turja et al., 2014). The IBR approach has also been widely used to investigate both native and caged bivalves in many studies (Leinio and Lehtonen, 2005; Campillo et al., 2013; Marigomez et al., 2013a, 2013b). In this study, IBR/n showed a significant difference among the sampling sites, with XZ site having the highest IBR/n value of 3.58. This reflected XZ as the most highly impacted site with the highest metal concentrations. On the contrary, the lowest IBR value (0.04) was obtained at PL site, indicating that this site was less-polluted. The present study represented the first stage of a monitoring program to evaluate the effects of metals on physiological functions of the oysters along the north coast of Shandong Peninsula. Future surveys will be conducted to evaluate seasonal variation in these biomarkers and allow the determination of baseline enzymatic activity levels in native oysters over a longer period of time. Overall, the assessment of combined multi-biomarkers is an effective tool to reflect water quality and identify the pollution components in a complex coastal environment. Data collected will thus provide a more comprehensive assessment of human-induced environmental risk and management decisions in coastal areas.

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