Integrated toxicity evaluation of metals in sediments of Jiaozhou Bay (China): Based on biomarkers responses in clam Ruditapes philippinarum exposed to sediment extracts

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\textbf{A B S T R A C T}

To evaluate the integrated toxicity of metals in sediments of Jiaozhou Bay, we exposed clam (Ruditapes philippinarum) to sediments extracts obtained using of sediment extraction with deionised water adjusted to pH 4 which simulated the weak acidity in the digestive juice of clams and tested the selected biomarkers responses in clams for exposure over 15 days. At the same time, the contents of metals in sediments were assessed with method of the mean sediment quality guideline quotient (SQG-Q). The integrated biomarker response version 2 (IBR\textsubscript{v2}) was used to assess the integrated toxicity induced by metals in sediment extracts based on biomarkers response in clams: the results demonstrated that site S7 located in the mouth of Nanxin'an River show higher IBR\textsubscript{v2} values compared to the other sites. The IBR\textsubscript{v2} values exhibited the good consistency with SQG-Q values.

\textbf{1. Introduction}

Significant quantities of metals from anthropogenic activities (river effluence, port development, shipbuilding, and mariculture, etc.) have been discharged into the marine environment in recent years, and are mainly deposited in sediments through the adsorption of suspended particulate matter (Yuan et al., 2012; Chaudhary et al., 2013). Metals accumulated in sediments can pose a threat to marine organism health and the equilibrium of a marine ecosystem once their contents exceed the corresponding threshold (Kalantzi et al., 2013). A variety of studies have demonstrated that sediments are a more effective reservoir of metals, as well as other pollutants, compared with the water column as they can record long-term changes in the environment, and thus metal contents in sediments are often used to evaluate the impact of anthropogenic activities on the estuarine and offshore environment (Long et al., 2006; Viguri et al., 2007; Pazi, 2011). However, the total amount of metals cannot characterise the toxicological and environmental hazards of sediments, because only those bioavailable metals have potential bio-toxicity (Luoma, 1989; Di Toro et al., 1990; Yu et al., 2012). In addition, the toxic effects of metals in the form of combined pollution on organisms cannot be simply obtained by chemical analysis (Tsangaris et al., 2011a, 2011b). Therefore, the assessment of environmental quality based on biological effects caused by pollution stress has attracted increasing research attention (Lehtonen et al., 2006; Lam, 2009).

A biomarker is signal indicator of abnormal changes at molecular and cellular levels when organisms suffer from external environmental stress (Depledge et al., 1995). A biomarker is sensitive to pollutants and also provided with an “early warning” function, and thus it has been considered as an effective tool for monitoring and assessing biological effects of pollutants (Nigro et al., 2006; Hylland et al., 2016; Bouhallouai et al., 2017). Furthermore, the biomarker assay was conducted as an important indicator of metals pollution in marine ecological health assessment by the International Council for the Exploration of the Sea (ICES) in 2007 (Galloway et al., 2007). According to the coexistence of various pollutants in the marine environment, the integrated biomarker response version 2 (IBR\textsubscript{v2}) was proposed by Sanchez et al. (2013) in the view of integrated biomarker response (IBR) proposed by Beliaeff and Burgeot (2002). This approach aims mainly to reflect the extent and severity of marine pollution from the simultaneous responses of multiple biomarkers to spot biocindicators, and meets the need for a comprehensive assessment of environmental
with a surface area of about 390 km², and forms a diversi-
ornament and clean seawater (De Domenico et al., 2011; Kerambrun et al.,
2012). Currently, the exposure culture way in most of previous studies
is that place bioindicators into a tank containing contaminated sedi-
ments, Jiaozhou Bay has been investigated by dozens of researchers
considering its important economic status and severely polluted status
in the last 30 years (Dai et al., 2007a, 2007b; Deng et al., 2010; Zhao
et al., 2016). In particular, large size, wide distribution, stable life, and strong
enrichment ability, as well as other characteristics of bivalves make
them have been widely used as bioindicators in various marine or-
organisms (Viarengo and Canesi, 1991; Moschino et al., 2012). Ruditapes
philippinarum, an important commercially mollusc, is widely distributed
in coastal waters of China and is also the main bioindicator used in the
Mussel Watch Programme (China), and thus it is suitable to be used as a
sentinel organism in this study (Meng et al., 2011).

Nevertheless, there is a crucial limiting factor that the availability of
bioindicators for studying area pollution assessment using biomarkers
(Bolognesi et al., 2004; Chariton et al., 2010). The target bioindicators
cannot always be collected in some sites because the density of marine
organisms in different areas varies, which cannot meet the require-
ments for biomarker determination. An effective method of solving the
aforementioned problem is to expose the previously prepared bioindici-
ators with the same physiological condition to sediments collected
from study area under laboratory controlled condition. At the same
time, this method can also eliminate the differences in non-pollution
factors (pH, salinity, temperature, etc.) between diverse sites
(Luedeking and Koehler, 2004; Holmstrup et al., 2010; Daftorn et al.,
2012). Currently, the exposure culture way in most of previous studies
is that place bioindicators into a tank containing contaminated sedi-
ment and clean seawater (De Domenico et al., 2011; Kerambrun et al.,
2012; De Domenico et al., 2013; Edge et al., 2014). A promising ex-
posure method is to expose the bioindicators to sediment extracts
(Amiard et al., 2007; Rigaud et al., 2012). The sediment extracts were
prepared using a chemical extractant which simulated the digestive
assimilation of particulate substances and was adjusted to physiological
pH. Moreover, metals in sediments can be more easily absorbed by
bioindicators compared with the original exposure method because
they are transferred into the liquid phase after treatment by the ap-
propriate extractant: this is helpful when enhancing the response of
bioindicators to different pollution sites (Rigaud et al., 2012). Amiard
et al. (2007) reported that metals accumulated in organisms were signi-
cantly correlated with the desorbed metals from sediments treated with
sediment extractant with buffers adjusted to pH 4 for Mytilus edulis
or pH 6 for Crassostrea gigas.

Jiaozhou Bay, located in the southern Shandong Peninsula and en-
circled by Qingdao City (China), is a typical semi-enclosed shallow bay
with a surface area of about 390 km², and forms a diversified complex
ecosystem in combination with the surrounding terrestrial environ-
mental (Fu et al., 2007; Liu et al., 2008). Jiaozhou Bay is also an im-
portant economic zone for mariculture, the salt-production industry,
and tourism in the sea of northern China (Gao et al., 2003). Never-
theless, with booming urbanisation and industrialisation around the
coast of Jiaozhou Bay, a large number of pollutants such as metals were
discharged into bay via several seasonal rivers (Shi et al., 2011). Ac-
cording to the statistics of the official agency, there were > 22.6 tons of
metals and metalloid discharged into Jiaozhou Bay from streams in
2013 (Oceanic and Fishery Administration of Qingdao (OFAQ), 2015).
As for distribution, sources, and ecological risk assessment of metals in
sediments, Jiaozhou Bay has been investigated by dozens of researchers
considering its important economic status and severely polluted status
in the last 30 years (Dai et al., 2007a, 2007b; Deng et al., 2010; Zhao
et al., 2015; Lin et al., 2016; Xu et al., 2016). However, the study on the
integrated toxicity of metals in sediments based on multi-biomarker
responses has not yet been reported. In the current study, the clams R.
philippinarum were exposed to metals extracts obtained from sediments
of Jiaozhou Bay for 15 days, and the response characteristics of selected
bioindicators in clam digestive glands were also investigated, including
metallothioneins (MTs), glutathione S-transferase (GST), 7-ethoxy-
resorufin O-deethylase (EROD), and malondialdehyde (MDA), as well as
lysosomal membrane stability (LMS) and micronucleus frequency
(MNF) in haemocytes. Eventually, the integrated toxicity of metals in
the sediments of Jiaozhou Bay was evaluated using of calculated IBRv2
index based on biomarker responses.

2. Material and methods

2.1. Study area and sample collection

Jiaozhou Bay lies 35° 58′ to 36° 18′ N and 120° 23′ to 120° 23′ E and
is part of the Yellow Sea. The coast of Jiaozhou Bay is dominated by silt
and rocky coasts. There are various types of sediments in the study area,
and the sedimentary environment exhibits strong reducibility (Dai
et al., 2007a, 2007b). The tidal current in Jiaozhou Bay shows the characteristics of reciprocating flow and standing waves; the tide is a
regular semidiurnal tide and its Significant Wave Height (SWH) is <
5 m on the whole (Chen et al., 2012). Several small seasonal rivers with
various water, and sediment, loads discharge into the Jiaozhou Bay,
namely, Haibo, Licun, Baisha, Moshui, Hongjiang, Dagu, and Nanxin' an
Rivers (Fig. 1).

In the current study, site selection was mainly based on a previous
study of metals contamination and feature of major anthropogenic

Fig. 1. Map of the sediment sampling sites in Jiaozhou Bay.
stressors. The sediment samples were collected from eight sites in Jiaozhou Bay in April 2015 (Fig. 1). In detail, sites S1 to S5 and S7 were located at the mouth of Haibo River, Licun River, Moshui River, Hongjiang River, Dagu River, and Nanxi'an River, respectively; site S6 was in the centre of the bay, while site S8 situated in the mouth of bay. According to the spot investigation, sites S1 to S4 were primarily influenced by wastewater discharge from a sewage treatment plant, industrial sources, and agriculture; site S5 was close to an aquaculture area; site S6 was in a relatively less-impacted area; only a small amount of domestic sewage was emitted at site S8; site S7 was near Qianwan Port, which is the one of the largest ports serving Qingdao City.

At each site, triplicate surface sediment (0–5 cm) samples were collected use of a Van Veen stainless steel grab sampler with an area of 0.02 m², which were then mixed them into a uniform, composite sample. All samples were placed into polyethylene bags which had been treated with nitric acid solution (1:3, v/v) and transported to the laboratory in a cooled box (with ice packs) as soon as possible. Collected samples were stored at −20 °C in a refrigerator until further analysis. It is worth mentioning that few bivalve organisms were found in sediments at site S5.

2.2. Determination of metals contents in sediments

On the basis of the Specification for Marine Monitoring (GB 17378.5-2007) (State Ocean Administration of China (SOA), 2007), the sediment samples were dried at 105 °C to a constant mass for analysis of As, Cd, Cr, Cu, Pb, and Zn; and the samples for detecting Hg were only air-dried by exposure to ambient air. All the sediment samples were then ground into powder-form and sieved through a 63 μm square aperture mesh, followed by homogenisation, to obtain consistent physical properties. The ~0.2500 g of pre-treated samples was digested with 12 mL aqua regia (HNO₃:HCl (3:1, v/v)) in closed Teflon digestion vessel with a microwave digestion instrument (GFAAS; M6 Series, Thermo Scientific, USA), the mixture was kept at 120 °C for 10 min and 180 °C for 25 min and then unclosed and heated on electric heating plate at 130 °C until it evaporated to coarse-dryness. Following cooling, the remaining digested samples were brought to a final volume of 45 mL using 2% HNO₃ solution (v/v). The trace element contents (As, Cd, Cr, Cu, Pb, and Zn) were determined by graphite furnace atomic absorption spectrophotometry (GFAAS; M6 Series, Thermo Scientific, USA); while a cold-vapor atomic fluorescence spectrometry (AFS-920; Beijing Titan Instruments Co., China) was used to analyse Hg and As. All metals contents in sediments were presented as mg kg⁻¹ (dry mass).

Quality control and quality assurance were determined using of reagent blanks and standard reference materials (SRM, GBW 07314, supplied by the Second Institute of Oceanography, SOA, China). The differences in metals contents between the certified and detected values were found to be < 15%, and this indicated that the analytical results were accurate.

2.3. Assessment of metals in sediments

The threshold effects level (TEL) and probable effects level (PEL) (Table 1), proposed by Long et al. (1995), have been used widely to assess bio-toxic effects caused by single metal (Gao and Chen, 2012; Wang et al., 2015; Li et al., 2017). According to the values of TEL and PEL, metals contents in sediments were divided into three classes, i.e., rare, occasional, and frequent adverse bio-toxicity effects (Long et al., 1995). In consideration of the fact that metals are always present in sediments as complex mixtures, the method of mean sediment quality guideline quotient (SQG-Q) has been applied to evaluate the possible bio-toxic effect of combined toxicant groups by calculating mean quotients for a variety of metals using the following formula (Long and MacDonald, 1998):

\[
SQG-Q = \frac{1}{n} \sum_{i=1}^{n} \left( \frac{C_i}{PEL_i} \right)
\]

where \( C_i \) is the measured content of metal \( i \) in the sediment, PEL\(_i\) is the PEL value for metal, (Table 1) and \( n \) is the number of selected metals. According to the calculated SQG-Q values, the classes of adverse bio-toxicity level were defined as: SQG-Q ≤ 0.1: un-impacted or lowest potential for observing adverse bio-toxicity effects; 0.1 < SQG-Q < 1: moderate impact potential for observing adverse bio-toxicity effects; SQG-Q ≥ 1: highly impacted potential for observing adverse bio-toxicity effects (MacDonald et al., 2000).

2.4. Preparation of sediment extracts

Experiments were particularly designed to subject sediments in vitro to the physiological pH prevailing in the digestive gland of clam. The digestive juice of clam pH values exhibited weak acidity, and were mostly between 4 and 6 (Owen, 1966; Griscem et al., 2002). Furthermore, previous studies showed that metals in sediments were more easily released with a decrease in the pH of the surrounding environment (Atkinson et al., 2007; Amiard et al., 2007; Cabon et al., 2010). Therefore, for the sake of exploring the response of biomarkers to metals released from sediments to the utmost extent under the effect of digestive juice, the pH value of the extractant was set to 4 in this study. For preparation of sediments extracts, 1 g of air-dried collected sediment, sieved through a 63 μm square aperture mesh, was dispersed into 80 mL of deionised water (18.3 MΩ·cm) adjusted to a pH of 4 (using 1 M NaOH and 1% acetic acid (v/v)). A mixture of deionised water and sediments were stirred slightly for 12 h at ambient temperature (25–27 °C), and then the sediment extracts were recovered by centrifugation (3000g for 10 min). All extractions were carried out in duplicate.

2.5. Sediment extracts exposure

*R. philippinarum* with an average length of 3.5 ± 0.4 cm were provided from the culture zone of Hongdao Sea (Qingdao, China) and acclimated for 14 days in clean seawater under controlled conditions of water temperature (18 ± 1 °C), pH (8.0), salinity (30‰), and light: dark regime (12:12 h). During this procedure, the clams were fed with suspensions of the green algae *Chlorella pacifica* (1.3 × 10⁷ cells/L per day) every day with regular replacement of the contents of the tank by clean seawater. At the same time, an air pump was used for uninterrupted aeration to keep a constant level of dissolved oxygen (6 ± 0.5 mg L⁻¹).

For the exposure experiments, clams were randomly assigned into group of 20 per glass cylinder (10 L) and then treated with 2 L clean seawater or sediment extracts at each site. Both the clean seawater and sediments extracts were adjusted to a pH of 8 and salinity of 30‰ before exposure, and were changed daily in each glass cylinder. The lighting conditions, feeding of the bait, and the maintenance of the dissolved oxygen level were consistent with the conditions for clam domestication. A certain number of clams were taken from each cylinder to determine the biomarkers of haemocytes and digestive gland after exposure for 15 days at 18 °C. Each exposure group carried out in triplicate.

Table 1

<table>
<thead>
<tr>
<th>Metal</th>
<th>TEL</th>
<th>PEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg</td>
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<td>0.70</td>
</tr>
<tr>
<td>Cd</td>
<td>0.68</td>
<td>4.21</td>
</tr>
<tr>
<td>Pb</td>
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<tr>
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<td>124</td>
<td>271</td>
</tr>
<tr>
<td>As</td>
<td>7.24</td>
<td>41.6</td>
</tr>
</tbody>
</table>

2.6. Sample pre-treatment and biomarkers assay

2.6.1. LMS assay

LMS was detected according to the method described by neutral red retention (NRRT) (Lowe et al., 1995). The method was based on the fact that neutral red dye can reduce the lysosomal membrane stability of haemocytes, and make neutral red dye leak into lysosomal membrane cytoplasm to cause the cytoplasm to turn red. Haemolymph (1 mL) was obtained from the adductor muscle of R. philippinarum with a 1 mL hypodermic syringe and then transferred to pre-cooled centrifuge tube. The haemolymph was gently mixed by similar volume of 0.9% NaCl solution (v/v), from which a 40 μL sample was pipetted onto the centre of a microscope slide. The microscope slide was placed in humid chamber for 3 min in order to make the haemocytes adsorb to the slide. Following incubation, redundant liquid was removed from the slide and 40 μL of neutral red solution (Sigma) was added and a cover slip also applied. Results of lysosomal membrane stability was visually examined every 15 min for 1 h and then every 30 min for 2 h by light microscope (×400 magnification). The assay was terminated and the time (min) recorded when > 50% of the haemocytes leaked the neutral red dye out of the lysosome into the cytoplasm.

2.6.2. MNF assay

MNF was measured according to the method of Baršené et al. (2004), and its testing principle was based on haemocytes stained with Giemsa dye can produce micronuclei. Approximately 300 μL of haemolymph was sampled from the posterior adductor muscle of each clam and then smeared evenly on the microscope slide which pre-treated by polylysine (Sigma). The slide was then transferred to humid chamber keep 30 min to enable the cells to adhere to the slides. Redundant liquid was removed and the adhered cells were fixed in 1% formaldehyde (v/v) for 15 min, and then the slide was left to air-dry overnight. Following fixation, the slide was stained by 10 drops of 5% Giemsa dye (v/v, Sigma) for 15 min and rinsed with distilled water twice to remove excess Giemsa dye. Subsequently, the stained slide was air-dry and then covered and fixed with cover slip and neutral balsam. Finally, the MNF of haemocytes was measured under microscope (×1000 magnification). A total of 1000 cells were observed for each sample, and the MNF was expressed as the number of micronuclei per 1000 cells scored (%).

2.6.3. Preparation of digestive gland extracts

The whole procedure was kept at 4 °C. Digestive gland, the detoxification organ of clams, from each exposure treatment was divided into two sections. For measurement of MTs, the samples was homogenised (1:5, w/v) with cold Tris–HCl buffer (0.02 M, pH 8.6) that consisted of 0.5 mM phenylmethylsulfonylfluoride, 0.01% β-mercaptoethanol, and 0.5 M sucrose. Samples were centrifuged (25,000g at 4 °C for 20 min) and the supernatant collected for MTs testing. The other part of the sample was used to test for the protein content and the other biomarkers (except for MTs). The sample was homogenised (1:4, w/v) of cold Tris–HCl buffer. The homogenate was centrifuged (10,000g, for 15 min at 4 °C) and the supernatant used for biochemical determination.

2.6.4. Protein assay

The protein content was analysed following the Coomassie Brilliant Blue (CBB) method (Bradford, 1976) and anions of CBB dye can combine with –NH₂ in the protein molecule to turn the solution blue. The 50 μL of pre-treated tissue homogenate was placed into centrifuge tube and then 3.0 mL of CBB dye was added. The extracts and dye were mixed gently until the mixture become uniformity, and this mixture was kept for 10 min to enable it react completely. Following settling, the preparation was measured using spectrophotometer (Spectronic 200, Thermo Scientific, USA) at a wavelength of 595 nm. The protein content can be calculated according to absorbance and the results data were presented as g L−1.

2.6.5. EROD assay

EROD activity was determined activity was determined following the method described by Burke and Mayer (1974). The reaction was initiated by adding 10 μL tissue homogenate, 950 μL PBS buffer (pH 7.4), 10 μL 7-ethoxyresorufin (0.1 mM) and 30 μL NADPH-Na solution (1 mM) to the microsamol fraction, following incubation at 20 °C for 5 min. Subsequently, 200 μL formaldehyde solution was added in order to terminate the reaction. The 7-hydroxyresorufin fluorescence was determined at 550 nm/580 nm excitation/emission wavelengths using DTX Multimode plate readers (Beckman Coulter, Inc., USA). A standard curve was measured by resorufin sodium salt as standard. The resulting data were expressed as pmol of conjugated product formed per minute and per milligram of protein (pmol min−1 mg−1 protein).

2.6.6. GST assay

GST activity was tested in post-mitochondrial supernatant (PMS) (at 25 °C) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate basing on the method of Habig and Jakoby (1981). The assay mixture included 100 μL tissue homogenate, 2.60 mL of phosphate buffer (0.2 M, pH 7.9), 100 μL of GSH (30 mM) and 100 μL of CDNB (30 mM). The reaction was started by the addition of 100 μL of PMS, and the increase in absorbance was recorded at 340 nm by DTX Multimode plate readers. The GST activity was calculated as nmol CDNB conjugate formed min−1 mg protein using a molar extinction coefficient of 9.6 × 10³ M−1 cm−1.

2.6.7. MDA assay

MDA content, a biomarker of lipid peroxidation, was evaluated using a commercial kit (Nanjing Jiancheng Bioengineering Inc., China). The assay mixture consisted in 200 μL tissue homogenate and 2.0 mL 6% thiobarbituric acid (TBA) solution in 99% acetic acid (v/v). The assay mixture was then incubated in hot water (95 °C) bath for 40 min. The samples were allowed to cool at room temperature and centrifuged 6000g for 10 min. MDA produced during lipid peroxidation can react with TBA to form red products. The red colour produced was tested at 532 nm by spectrophotometer, and the resulting data were presented in nmol mg−1 protein.

2.6.8. MTs assay

MTs content was determined by the spectrophotometric method of Viarengo et al. (1997a, 1997b). Three volumes of absolute ethanol (−20 °C) were added to the supernatant resulting from ethanol/chloroform extraction to precipitate the MTs. The MTs pellets were resuspended in NaCl/HCl/EDTA, respectively, to remove metal cations still combine with the MTs. After this Ellman reagent (pH 8.0 phosphate buffer containing DTNB) was added to the solution. The DTNB can react with the thiol (−SH) groups of MTs and the contents of −SH can indirectly measure the MTs level in tissue. The −SH contents was analysed at 412 nm with spectrophotometer, and the MTs contents' data were expressed as μmol-SH g−1 protein. A standard curve was measured by GSH as standard.

2.7. Integrated biomarker responses version 2 (IBRv2) calculation

In this study, “integrated biomarker response version 2” (IBRv2) proposed by Sanchez et al. (2013), was used to evaluate the integrated biomarkers response of treated group compared to a control group. To get IBRv2 values, the mean of an individual biomarker (Xi) was compared to the mean of reference data (Xi0), which in our research were the biomarker values of the clams exposed to clean seawater without sediment extracts. Then a log transformation was performed to reduce the variance: Yi = log (Xi/Xi0). In the next step, the general mean (M) and standard deviation (S) were calculated as previously described by Belaief and Burgeot (2002), and Yi is standardised as Zi = (Yi – M)/S. To create a basal line centred on zero and to represent biomarker variation according to the basal line, the mean of the standardised biomarker response (Zi) and the mean of the reference biomarker data
(\(Z_0\)) were applied in defining the biomarker deviation index (\(A\)), \(A_i = Z_i - Z_0\). Finally, the absolute value of \(A_i (|A|)\) of every biomarker was computed for each of site to get the IBR\(_{v2}\) as IBR\(_{v2} = \Sigma|A|\). A parameter is plotted in a star plot to represent the reference deviation of each evaluated biomarker.

2.8. Statistical analysis

All metals contents in sediments and each biomarker activity (or content) of calms were detected in triplicate. All data were expressed as mean ± SD. The significant differences in biomarker responses of different exposure groups were examined using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The relationship between IBR\(_{v2}\) and SQG-Q values was assessed by Pearson's correlation analysis. A significance level of all statistical tests was regarded as \(P < 0.05\). All statistical analyses were conducted using MS-Excel2007® and SPSS 19.0 software.

3. Results and discussion

3.1. Metals pollution in sediments

All selected metals contents in the surface sediments from Jiaozhou Bay at each site are shown in Fig. 2. The content of Hg ranged from 0.12 (S6) to 0.58 (S1) mg kg\(^{-1}\). As from 9.1 (S1) to 20.77 (S7) mg kg\(^{-1}\), Zn from 66.8 (S6) to 243.4 (S7) mg kg\(^{-1}\), Cu from 12.0 (S6) to 124.5 (S7) mg kg\(^{-1}\), Pb from 42.0 (S8) to 93.1 (S7) mg kg\(^{-1}\), Cr from 83.3 (S4) to 140.6 (S3) mg kg\(^{-1}\), and Cd from 0.08 (S6) to 0.37 (S8) mg kg\(^{-1}\). Sediment obtained from S7 showed generally higher metals contents compared with the other sites, while site S6 exhibited relatively lower metal contents. All metals contents were below the corresponding PEL values with exception of Cu at sites S7 and S8; nevertheless, the TEI values were exceeded by As, Cr, and Pb at all sites, by Hg and Cu at seven sites (apart from S6), and Zn at sites S2, S3, and S7. This phenomenon indicated adverse bio-toxicity effects on biota are occasionally observed in all sampling sites. As shown in Table 2, the calculated SQG-Q values of different sediments decreased thus: S7 (0.68) > S3 (0.54) > S1 (0.43) > S8 (0.41) > S5 (0.40) > S2 (0.39) > S4 (0.35) > S6 (0.27). The SQG-Q values for all sediments fall into interval of 0.1–1.0, indicating that moderate impact potential adverse bio-toxicity effects are observed in all sampling sites in the study area.

3.2. Biomarker responses

3.2.1. LMS

LMS, expressed as the neutral red retention time (NRRT), can precisely reflect the changes of the organisms suffered from external stress at the cellular level (Moore et al., 2006; D’Agata et al., 2014; Martínez-Gómez et al., 2015). Generally, the greater damage of the integrity of the cell function, the smaller the NRRT becomes (Lowe et al., 1995). Previous studies have proved that LMS is a sensitive biomarker to metals because stress caused by metals can result in a decrease of NRRT, and thus it was widely used for monitoring and evaluation of metals in the marine environment (Brooks et al., 2015; Løf et al., 2016; Balbi et al., 2017). Furthermore, LMS is a non-specific stress response to contaminants, and is thus considered to be the most reliable biomarker in water quality assessment (Edge et al., 2014).

As shown in Fig. 3(a), the lysosomal membrane stability of clam haemocytes showed significant differences in different exposure groups for 15-days exposures. The NRRT of all treated groups was significantly lower than control group (\(P < 0.05\)). The minimum NRRT appeared in site S7 extract (23 min), while maximum NRRT was observed in site S6 extract (62 min). This lysosomal membrane instability phenomenon also existed in previous studies on Mytilus edulis exposed to metals Cu, Cd, and Hg (Koukouziouka and Dimitriadis, 2008). According to the criteria for cytology adversity grading basing on LMS proposed by Aguirre-Martínez et al. (2013), clams remained healthy if NRRT was > 80 min; they were identified as stressed but can be restored if NRRT was between 45 and 80 min; once the NRRT is below than 45 min, the clams are subjected to severe stress and may produce pathological characteristics. In the current study, the NRRT of sites S1–S3 and S7 extracts were down to 45 min. Thus it can be seen that these sediment extracts may cause diminished harm to clams. The NRRT in other sites varied from 48 min to 62 min, indicating that clams exposed to these sediment extracts were not yet seriously affected.

3.2.2. MNF

DNA is the carrier of genetic information, and any changes in its structure can cause serious biological changes. After exposure to genetic pollutants, organisms will suffer hereditary damage manifested as an MNF increase (Çakal et al., 2015). Hence, MNF is considered an important tool for genetic toxicity assessing and monitoring (Viarengo et al., 2007). Micronucleus technology is based on genetics on the principle of chromosome aberration established by the degree of damage to the chromosome to characterize surrounding environmental pollution (Stefania et al., 2009; Arslan et al., 2010; Tsangaris et al., 2011a, 2011b).

Fig. 3(b) showed the changes of MNF in clams *R. philippinarum* haemocytes after exposure to sediment extracts for 15 days. The MNF of all treated groups was significantly induced (\(P < 0.05\)) compared with the control group. The MNF peaked at sites S7 and S1 and was higher than that in the control group by 6.92 and 5.73 times, respectively. In contrast, MNF at sites S5 and S6 was lower, showing that all sediment extracts can produce hereditary damage to clams, and site S7 together with S1 conferred higher genetic toxicity. Some scholars also have confirmed that metals pollution can cause the significant increase of the MNF in haemocytes of clams, for example, Bolognesi et al. (2004) found MNF of *Mytilus galloprovincialis* haemocytes had a significant correlation (\(P < 0.05\)) with the Hg content in their tissues after 30 days of exposure in the Ligurian coast (Italy).

3.2.3. MT contents

Metallothioneins (MTs) are a class of naturally-occurring intracellular non-enzymatic proteins. MTs are cysteine-rich, and play a key role in maintaining homeostasis of essential metal ions and resisting external metals in intracellular spaces (Capdevila and Atrian, 2011; De Domenico et al., 2011). Since MTs can be induced by metals, such assays have been widely performed on metals toxicity effects for early warning and assessment of metal pollution in marine environments (Amiard et al., 2008; Giacci et al., 2012; Guo et al., 2017).

As shown in Fig. 3(c), MT contents in clam digestive glands increased by 1.86 to 78.82% from that in the control group, and most of treated groups (except for site S4) had significant differences from the control group (\(P < 0.05\)). The most significant MT induction was found at S7 (23.31 µmol·SH g\(^{-1}\) protein) where the sediments were more severely polluted by metals (Table 2). An increase in MT content was also observed in the digestive gland in *Perna viridis* (L.) exposed to clean seawater containing higher concentration of Hg\(^{2+}\) (0.045 mg L\(^{-1}\)) for 15 days (Smaoui-Damak et al., 2009). All told, the degree of induction effect of MTs was quite different in different treated groups, and thus was suitable for the distinction of metals pollution levels at each site.

3.2.4. MDA contents

Reactive oxygen species (ROS) accumulated under stress can attack polyunsaturated fatty acids (PUFA) on cell membranes. This process can trigger lipid peroxidation (LPO) to form lipid peroxides, which is a typical marker of oxidative damage to cells (Company et al., 2004; Xia et al., 2017). In particular, MDA content is regarded as a pivotal indicator of lipid peroxidation because it is a major oxidation product of PUFA (He et al., 2012; Xia et al., 2016).

The MDA contents of all groups after exposed to 15 days are shown...
Similar to MTs, MDA accumulation in clams treated with sediments extracts differed significantly from the control group ($P < 0.05$), and also presented a tendency to be induced. The increase in MDA content often indicates that the self-repairing ability of the antioxidant defence system of the organism is not sufficient to eliminate the negative effects caused by external pollution, and the oxidation-reduction equilibrium in the body has been destroyed (Taylor and Maher, 2012). In detail, MDA contents in treated groups ranged from 10.71–13.90 nmol mg$^{-1}$ protein and the highest value was recorded in site S3 extract with its greater metals pollution load. Pytharopoulou et al. (2008) reported MDA contents in gills of caged mussels (*Mytilus galloprovincialis*) exposed to a heavily polluted site by metals in Gulf of Patras (Greece) were higher than when exposed to a relatively clean site. Vlahogianni and Valavanidis (2007) also found that metals can result in a significant increase in MDA contents of the *Mytilus galloprovincialis* digestive gland after 10 days of exposure.

### 3.2.5. EROD and GST activity

The detoxification of exogenous pollutants process in organism was divided into Phase I (biotransformation) and Phase II (conjugation) stages. Biotransformation phase I enzyme EROD, an isoenzyme encoded by the P450 sub-enzyme 1A, has been considered as an important
Table 2
PEL-Q and SQG-Q values of metals in sediments obtained from Jiaozhou Bay.

<table>
<thead>
<tr>
<th>Sites</th>
<th>PEL-Q</th>
<th>SQG-Q</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hg</td>
<td>As</td>
</tr>
<tr>
<td>S1</td>
<td>0.83</td>
<td>0.22</td>
</tr>
<tr>
<td>S2</td>
<td>0.66</td>
<td>0.24</td>
</tr>
<tr>
<td>S3</td>
<td>0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>S4</td>
<td>0.70</td>
<td>0.27</td>
</tr>
<tr>
<td>S5</td>
<td>0.26</td>
<td>0.45</td>
</tr>
<tr>
<td>S6</td>
<td>0.17</td>
<td>0.35</td>
</tr>
<tr>
<td>S7</td>
<td>0.74</td>
<td>0.50</td>
</tr>
<tr>
<td>S8</td>
<td>0.19</td>
<td>0.33</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>0.52 ± 0.27</td>
<td>0.33 ± 0.10</td>
</tr>
</tbody>
</table>

Fig. 3. Biomarker activity (or content) in the *R. philippinarum* at each exposure group: (a) lysosomal membrane stability (LMS); (b) micronucleus frequency (MNF); (c) metallothioneins (MTs); (d) malondialdehyde (MDA); (e) 7-ethoxyresorufin O-deethylase (EROD); (f) glutathione S-transferase (GST). CK represents control group; (a), (b), (c), (d), (e), (f) present significant differences (*P* < 0.05) compared to CK.
biomarker for metals pollution (He et al., 2012; Zheng et al., 2016; Cao et al., 2017). The inhibition of EROD activity caused by metals may be in two ways: (1) metal ions can make inactivation of EROD by combining with their $-SH$; (2) metal ions can produce a large amount of ROS in the organism which thus preventing the expression of P450 subenzyme 1A genes (Benedetti et al., 2007). A large number of studies have shown that EROD activity in clam digestive glands is significantly inhibited when exposed to Hg$^{2+}$, Pb$^{2+}$, and other metals (Faria et al., 2009; Zhang et al., 2010). In agreement with these findings above, EROD activity was inhibited in all treatment groups, and also differed significantly from the control group in this study ($P < 0.05$) (Fig. 3(e)). The inhibition rates of EROD activity in all treated groups was ranged from 37.22–63.05% as compared with control group, and the most significant suppression effect was observed at sites S1 and S7 with their higher SQG-Q values.

GST, a related biomarker with EROD, has been considered as an important conjugation phase II enzyme in organisms which can catalyse the combination of phase I metabolites with GSH, and thus is beneficial to exclude toxic substances from the body (Antonino et al., 2015). The significantly decreased GST activity in clam digestive gland was observed in most treated groups with exception of sites S1 and S8 extracts after 15 days exposure ($P < 0.05$) (Fig. 3(f)). The strongest inhibitory effect in GST activity was recorded in site S5 extract with a decline of 63.65% compared to the control group. The depressed expression of GST often predicts that the detoxification mechanism has been severely damaged (Cappello et al., 2013). In agreement with our findings, a number of recent studies reported that GST activity of calms exposed to pollution adversity (metals, waste water, etc.) was also inhibited (Verlecar et al., 2008; Rola et al., 2012; Tlili et al., 2013). Moreover, Kamel et al. (2012) found GST activity of digestive glands in *Ruditapes decussatus* after exposure to treated municipal effluent shows a “bell-type” response with the characteristic of increasing after initially declining. Generally speaking, the increase of GST in the early stages of exposure can catalyse reduced Glutathione (GSH) to remove metals ions (Cappello et al., 2013); however, the effect of sediment extracts on GST activity was inhibited in the current study, which may be due to the accumulated metals ions in digestive gland inactivate enzymes by binding to their $-SH$ (Viarengo et al., 1997a, 1997b).

### 3.3. Integrated biomarker response version 2 (IBR$_{2}$)

It is almost impossible to measure environmental stress accurately by using a single biomarker (Wang et al., 2011). For the sake of comprehensively assessing the all-round stress of sediment extract pollution induced in clams, the IBR$_{2}$ index was computed in this study. This approach can combine several biomarkers to an individual value to evaluate the risk state in laboratory and field studies (Sanchez et al., 2013; Bebianno et al., 2015; Carbajal-Hernández et al., 2017; Sanchez-Hernandez et al., 2017). The IBR$_{2}$ index of each treated group, as well as SQG-Q values deriving from sediments, were shown as star plots and shown in (Fig 4(a), (b), and (c)). In general, the IBR$_{2}$ values spatially change across different sediment extracts within the range 10.57 to 18.42. Based on this index, the grade of the extracts according to the degree to which they were influenced can be ranked as: S7 > S1 > S2 > S3 > S8 > S5 > S6 > S4. The IBR$_{2}$ value for site S7 extract was markedly higher than those obtained from other sediment extracts.

![Fig. 4. Star plots of integrated biomarker responses version 2 (IBR$_{2}$), mean sediment quality guideline quotient (SQG-Q), and biomarkers. (a): IBR$_{2}$ values for each site sediment extract; (b): SQG-Q values for each site sediment; (c): biomarkers star plots for each site sediment extract.](image-url)
which indicating that site S7 suffered the most serious metals stress in the study area. Similarly to our result, previous study also confirmed that metals in sediments near this area pose a higher ecological risk than other regions of Jiaozhou Bay due to its proximity to Qianwan Port which is mainly used to carry iron ore and coal (Lin et al., 2016). Sites S4 and S6, located at the mouth of Hongjiang River and the centre of Jiaozhou Bay, respectively, and their extracts, exhibited the lower IBRv2 values. The IBRv2 values of the sediment extracts from the remaining sites showed a smaller difference.

The pattern of star plot of IBRv2 is similar to the SQG-Q and Pearson correlation analysis in that it also indicated an extremely strong correlation between IBRv2 and SQG-Q values (R = 0.905, P < 0.01). These results confirmed that the pollution pressure in sediments caused by metals was consistent with biomarkers responses in digestive gland of clams. Si et al. (2016) calculated IBRv2 using seven biomarkers in SD rats to assess ground water contamination in Baotou City (China), and found that the IBRv2 index showed a significant correlation with the Nemerow composite index evaluated by ground water assay (P < 0.01). Marques et al. (2016) investigated metal accumulation and oxidative stress responses of two benthic species (Cerastoderma edule and Nephys hombergii) collected from two sites with distinct contamination degrees in the Tagus estuary (Portugal): they found a significant difference between the two different contaminated sites basing on the IBRv2 index, and there also was a significant correlation between IBRv2 values and metals contents in organisms (P < 0.05); however, it is worth mentioning that there are some small differences between the rank of sites obtained from SQG-Q and IBRv2 values. The reason for this was that SQG-Q values are calculated by total metals content in the sediments, while IBRv2 values are computed based on biomarker responses of R. philippinarum exposed to sediment extracts containing only part of the metals present in the sediments deemed bioavailable to clams. Conspicuously, the IBRv2 index was more appropriate for the qualitative, and not for the quantitative, evaluation of the area suffering different degrees of pollution (Lam and Gray, 2003; Serafim et al., 2012). Hence, the combination of biological effect (such as biomarkers) and traditional chemical quantitative monitoring will become commonplace in environmental assessment of the integrated toxicity of metals (Meng et al., 2013).

4. Conclusions

This study was the first time to adopt a multi-biomarker approach, IBRv2 index, to assess the integrated toxicity of metals in sediments of Jiaozhou Bay (China) based on biomarker responses in R. philippinarum exposed to sediment extracts. The selected biomarkers in the digestive gland of clams were detected and showed an obvious response to sediment extracts with different degrees of contamination after exposure for 15 days. The results of IBRv2 showed that the integrated toxicity of metals in site S7 extract was the highest of all sites, while sites S4 and S6 extracts exhibited lower heavy-metal stress. The IBRv2 values had excellent consistency with the SQG-Q values obtained from metals in sediments, and thus this approach could be regard as a sensitive tool with which to evaluate metal-induced stress over a large spatial scale environment such as Jiaozhou Bay. Consequently, this study can provide valuable information for environmental management and metals pollution control in Jiaozhou Bay.

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