Assessment of Organic Contamination along the Coast of Laizhou Bay, China: Combining Chemical Analysis and Integrated Biomarker Responses in the Clam *Ruditapes Philippinarum*

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Abstract

The marine coastal area is facing serious challenges due to several artificial pollution sources. An investigative biomonitoring survey was conducted along the coastal area of Laizhou Bay, China, which combined an evaluation of organic pollution and biomarker responses in the clam *Ruditapes philippinarum*. Meanwhile, the contents of polychlorinated biphenyls (PCBs), total petroleum hydrocarbons (TPHs) and nonylphenol (NP) in surface sediments at the biomonitoring sites were measured. The results indicated that a complex mixture by these toxic organic pollutants was prevalent throughout the entire area. Concentrations (mean ± standard error) of PCBs, TPHs and NP in sediments of the study area were 1.90 ± 0.10 μg kg^{-1}, 39.55 ± 2.42 mg kg^{-1}, 9.23 ± 0.41 μg kg^{-1} dry weight, respectively. Biomarker responses of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Glutathione S-transferase (GST), total glutathione (GSHt) and lipid peroxidation (LPO) levels were assayed in the gills and digestive glands of *R. philippinarum* collected from eleven sites. Finally, biomarker responses obtained in gills were selected to calculate the integrated biomarker response (IBR) index and to assess the impact of integrated organic contaminants from different stations. The site S2 exhibited an IBR value of 2.65 and was considered as the place with the greatest stress in coastal environment. The western coast (S1-S4) and eastern coast (S9-S11) exhibited higher environmental stress than the sampling sites along the southern coast of Laizhou Bay. PCBs and NP were the main organic pollutants which have caused high level of pollution pressure on *R. philippinarum* in Laizhou Bay coastal area. The integrated assessment approach of organic contamination which combined chemical analysis and multi-biomarker responses was proved to be practical and useful in coastal environment assessment programs.

Introduction

Coastal ecosystems, including the coastal basin and the adjacent shore land, are among the most extensively modified and threatened ecosystems on account of urban development, industrialization and fishing (Cravo et al. 2012). Complex mixtures of pollutants are continuously released into these systems, deteriorating both the water and the sediment quality (Hong et al. 2005; Kueh and Lam 2008; Wurl and Obbard 2006). Amongst the known contaminations, hazardous organic compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), total petroleum hydrocarbons (TPHs), as well as nonylphenol (NP), a typical kind of endocrine disrupting chemicals (EDCs), are of great concern. Due to the characteristics of persistence, carcinogenicity, mutagenicity and estrogenic effects, these organic pollutants usually increase ecological disruption and risk (Jones and De Voogt 1999).

Sediments are known as major sinks for organic contaminants in aquatic environments. Study of sediments is an important step in mapping possible pollution sources and exposure pathways that make pollutants bio-available to benthic organisms (Pan et al. 2010). However, the assessment of organic contamination only by chemical analyze cannot reflect the integrated stress of these bio-toxicants on organism. Therefore, the method by using biochemical responses of many kinds of benthic organisms,

called biomarkers, was developed. Since the development of the “Mussel Watch” program in 1975, biological monitoring approach has been strongly advocated (Blair 2001; Pollard and Huxham 1998). A multiple biomarker approach achieves great development and has been widely applied to marine pollution assessment by using different organisms, such as fish and mussels (Baussant et al. 2009; Tsangaris et al. 2011; Turja et al. 2013). Compared with the single chemical monitoring method, biomarkers can provide information about the biological effects of pollutants rather than a mere quantification of environmental levels (Amiard et al. 2000), and the integration of biomarker responses with a certain stress index can provide an accurate evaluation on the health status of marine organisms and the marine environmental quality. Thereinto, the IBR index, proposed by Beliaeff and Burgeot (2002), is a practical graphic method using star plots to summarize biomarker responses to a single value reflecting the environmental stress of each study area. This method can effectively integrate various biomarker responses of general health, toxic effects and exposure to specific contaminants, and has been successfully applied to environmental pollution assessment in marine areas including the Mediterranean Sea, the Baltic Sea coasts, lagoon of Ria de Aveiro in Portugal and the Río Cuautitlán in México (Bodin et al. 2004; Broeg and Lehtonen 2006; Oliveira et al. 2009; Trujillo-Jiménez et al. 2011).

Up to now, a battery of biomarkers has been reported as indicators of contamination. Xenobiotics are capable of inducing oxidative stress in aquatic organisms by enhancing the intracellular reactive oxygen species (ROS) (Lesser 2006), while antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and non-enzymatic antioxidants (e.g., glutathione) are capable of rapidly scavenging ROS (Costa et al. 2012; Fernández et al. 2010; Fernández et al. 2012). Lipid peroxidation (LPO) and DNA damage occur when ROS production overwhelms antioxidant capability (Ahmad et al. 2004; Van der Oost et al. 2003). Biotransformation phase Ⅰ enzyme 7-ethoxyresorufin-O-deethylase (EROD) and phase Ⅱ enzyme Glutathione S-transferase (GST) activities have also been used as biomarkers to indicate exposure to PAHs and PCBs (Nesto et al. 2007; Page et al. 2004; Pathiratne and Hemachandra 2010). Changes in the levels of these biochemical endpoints are sensitive indicators of environmental disturbances, and a multiple biomarker approach, combined with chemical analysis, can provide a more efficient and useful evaluation of environmental hazards (Galloway et al. 2002).

The Laizhou Bay, located in the south of the Bohai Sea, ranges from the Qimu Cape to the Yellow River Estuary. The coastal area around Laizhou Bay has rich marine natural resources including biological, oil-gas, harbors and coastal tourism resources. In the past decades, industrialization and urbanization have developed rapidly around the bay, leading to the discharge of large amounts of domestic sewage and industrial effluents with various toxic organic pollutants into the bay. These pollutants cause considerable adverse effects on the marine environment, especially the coastal area which is the first to come into contact with artificial pollution sources (Gan et al. 2013). Those pernicious organic components put the marine organisms at risk and pose potential hazards to human health via consumption of marine animals.

Bivalves have been widely used as biomonitors in environmental assessment and monitoring programs due to their characteristics of filter-feeding habits, poor action ability and high accumulation capability for pollutants. The clam *Ruditapes philippinarum* has a widespread distribution in both estuarine and inshore marine environments of Laizhou Bay (Liu et al. 2017). Being of high nutrition and medical value, *R. philippinarum* is the main seafood resource for the local people and is of great economic importance in this area. In the present study, eleven sites along the Laizhou Bay coast were surveyed and *R. philippinarum* was selected as an indicator species. Our work aimed to investigate the level and spatial variation of toxic organic pollutants and to determine multiple biomarker responses of *R. philippinarum* in surface sediments along the Laizhou Bay coast. As an integrated approach, we employed the integrated biomarker response (IBR) in order to obtain better evidence of the existing impacts of selected contaminants and assess their environmental stress on the Laizhou Bay coastal benthic area.

Materials And Methods

Study area and sampling

The study was conducted along the Laizhou Bay coastal area. Reports of organic pollution research in this area are scarce considering the sediment environment. In this work, wild clams *R. philippinarum* at a shell length of 4.0 ± 0.5 cm (mean ± standard deviation) were collected from eleven stations along the coast of Laizhou Bay during the period of low tide in October of 2020 (Fig. 1). The sampling period was two months after the reproductive season. Surface sediment samples (0–10 cm depth) were collected at the same time using a stainless-steel grab sampler. Bottom water temperature, salinity, pH and dissolved oxygen (DO) were measured *in situ* (Table 1) by using a portable water analyzer (DZB-718, LEICI, USA).

Regarding each site, 300–500 clams were collected and transported to the laboratory in insulated boxes with ice inside on the premise to ensure that they were alive before dissection. The study protocol regarding clams was in accordance with national and institutional guidelines for the protection of human subjects and animal welfare. When the samples arrived, the dissection of the clams was performed. Firstly, 10 random individuals from each sampling site were selected for the evaluation of their physiological state, which was expressed by the condition index (Table 1). Condition index was calculated as the ratio of lyophilized dry weight of the soft tissues to the dry weight of shell. Dry weight of soft tissues and shells were obtained at -40°C during a maximum period of 48 h until their constant weight was achieved. The rest samples of *R. philippinarum* at each site were divided into two parts. For one part, gills and digestive glands of *R. philippinarum* were extracted for biomarker assays and were frozen at -80°C prior to analysis. For another part, soft tissue samples were obtained and pooled together, then were immediately frozen at -20°C until organic pollutant (PCBs, TPHs and NP) measurements. Three replicates of sediment (600 g) were sampled per each sampling site and were preserved at -20°C until further analysis. These sediments were used for organic matter content (PCBs, TPHs and NP) determination.

Table 1
Physical-chemical parameters measured in water and the condition index values (mean ± standard deviation, $n = 10$) for *R. philippinarum* collected from the sampling sites along the Laizhou Bay coasts.

Site	Temperature (°C)	Salinity	pH	DO (mg L^{-1})	Condition index
S1	24.1	27.3	7.32	5.25	8.2 ± 0.5
S2	23.3	29.2	8.25	8.65	11.2 ± 1.2
S3	24.2	27.7	8.11	8.37	10.4 ± 1.1
S4	29.0	30.5	8.45	8.46	9.6 ± 0.9
S5	27.5	30.2	7.68	7.15	9.6 ± 1.4
S6	25.4	24.2	8.32	9.02	10.1 ± 0.7
S7	30.1	27.3	8.43	8.03	11.1 ± 2.1
S8	32.2	28.5	7.72	7.94	11.1 ± 0.8
S9	27.8	29.4	7.28	7.25	9.7 ± 1.7
S10	26.5	23.5	7.25	7.02	10.6 ± 0.6
S11	31.2	30.2	8.21	8.90	10.3 ± 0.7

Chemical analysis in sediments and in R. philippinarum

Seven PCB congeners (PCB 28, 52, 101, 138, 153, 180, 194) were determined using capillary gas chromatography (GC 2010 AF, Shimadzu, Japan) according to the method described in the Chinese National Specification for Marine Monitoring (GB 17378 – 2007). The spiked recoveries of PCB mixed standards (ISO6468, Accustandard, USA) were 70–111% for sediments and 78–116% for *R. philippinarum*. A fluorometric spectrophotometry (F-4600, Hitachi, Japan) was used to determine the content of TPHs in the sediment and biological samples by using the method described by Okparanma and Mouazen (2013). The recoveries of the oil standard for sediment and *R. philippinarum* samples were 97–102% and 95–109%, respectively. NP in sediment was analyzed according to the method interpreted by Li et al. (2003), and NP in *R. philippinarum* was analyzed by utilizing the method described by Wang et al. (2007). The surrogate standard, p-*tert*-Butylphenol (MFCD00002367, Accustandard, USA), was spiked for quality control and its respective recoveries were 66–109% for sediment and 75–96% for *R. philippinarum*. Three replicates were performed for each measurement.

Biochemical Analyses

Homogenate preparation

Gills and digestive gland samples were homogenized in Tris–HCl buffer (20 mM, pH 7.8) (1:4, w/v). Half part of the homogenate was centrifuged for 15 min (6,000g) at 4°C and the supernatant was used for determination of total glutathione (GSHt) and LPO. The rest was centrifuged for 15 min (12,000g) at 4°C and the supernatant was used for SOD, CAT, GPx and GST assays.

Biomarker assays

SOD activity was determined following the method described by McCord and Fridovich (1969). CAT activity was determined by measuring the consumption of the H_2O_2 substrate at 240 nm according to Aebi (1974). GPx activity was measured by a modification method of Hafeman et al. (1974). GST activity was measured according to Habig et al. (1974). GSHt content was determined as the sum of the reduced and oxidized glutathione, which was determined according to the fluorometric method by Hissin and Hilf (1976). Thiobarbituric acid reactive substances (TBARS) were assayed to reflect the state of lipid peroxidation (LPO) of the membranes by using the method developed by Buege and Aust (1976). Protein content was determined at 595 nm by Bradford assay using bovine serum albumin as a standard (Bradford 1976). For each measurement, three replicates were performed.

Calculation Of Ibr

A method for combining all the measured biomarker responses into a general stress index, termed “integrated biomarker response (IBR)”, was applied to this study (Beliaeff and Burgeot 2002). n previously used as a tool for assessing environmental risk The procedure for the IBR calculation of each biomarker is: (1) calculation of the mean and standard deviation (SD) for each station; (2) standardization of the data for each station: $Y_i = (X_i - m_i)/S$, where Y_i is the standardized value of the biomarker, X_i is the mean value of a biomarker from each station, m_i is the mean of the biomarker calculated for all the stations, and S_i is the standard deviation calculated for the station-specific values of each biomarker; (3) calculation of the Z_i value via the equation $Z_i = Y_i$ or $Z_i = -Y_i$ on the condition that the biomarker is induced or inhibited. The score (B) for a given station is computed as $B_i = Z_i + |min|$, where $B \geq 0$ and $|min|$ is the absolute value of the minimum value in the dataset. Scores of all the measured biomarkers for each station are represented in a star plot and the IBR value of this station is calculated as the area of the star plot via the following formula:

$$IBR = \sum_{i=1}^{n} A_i / n$$

Where A_i is the triangular area represented by two consecutive biomarker scores (B_i, B_{i+1}) on the star plot, and n is the number of biomarkers used in the IBR calculation. In this study, the IBR value was divided by the number of biomarkers, as suggested by Broeg and Lehtonen (2006).

Statistical analysis

Statistical analyses were performed using the SPSS statistical package (ver. 17.0, SPSS Co., USA). The data were tested first for normality using Komogorov-Smirnov test, and then tested for homogeneity of variances using the method of Levene test to check whether they meet statistical demands. For data of organic pollutants, one-way analysis of variance (ANOVA) was applied to compare differences among sampling sites using the Tukey test or Games-Howell test. All differences were considered significant at $p<0.05$. Pearson correlation analysis and Spearman correlation analysis were performed in order to verify the relationship among pollutants, water parameters, biomarkers and IBR values.

Results And Discussion

Spatial distribution of organic pollutants

The mean concentrations of PCBs, TPHs and NP in surface sediments are presented in Table 2. The content of organic pollutants varied remarkably at different stations. The highest total PCB concentration was found in S4 sediments (6.56 μg kg^{-1}), followed by S10 (3.53 μg kg^{-1}), and the lowest values were detected in those from S5, S7 and S9 (from 0.68 to 0.74 μg kg^{-1}). The other sites showed a moderate PCB concentration which ranged from 1.01 to 1.93 μg kg^{-1}. The average content of total PCBs along the Laizhou Bay coastal area was 1.90 μg kg^{-1}. Compared with the levels found worldwide, the Black Sea (0.3–6.8 μg kg^{-1} dw), the Caspian Sea (0.03–6.4 μg kg^{-1} dw), Southwestern of Baltic Sea (0.1–11 μg kg^{-1} dw), Gulf of Alaska (0.1-2 μg kg^{-1} dw), Kara Sea (nd-1.5 μg kg^{-1} dw) (Pan et al. 2010), PCBs in Laizhou Bay sediments were in the same level as those reported in other coastal regions, but lower than the remote areas of Europe and North America.

In sediments of the Bohai Sea, different congener patterns were observed in different areas, and PCB28 and PCB52 were the predominant PCB congeners in sediments at S1, S2, S6, S8 and S11 (Fig. 2a). However, in samples collected in other sites, higher chlorinated PCBs including penta-PCB, hexa-PCB and hepta-PCB, accounts for more than 50% of total PCBs. The difference indicated that PCBs in the Laizhou Bay sediments might be from mixed contribution of various sources from different areas such as release from incineration, re-emission of previous industrial usage and unlawfully usage nowadays (Wang et al. 2015).

The sediments from S5 presented the highest TPHs concentration (285.96 mg kg^{-1}), which was 8-165 folds of those in all other sites (from 1.75 to 35.45 mg kg^{-1}). S5 is near Weifang port, a medium port in China, which is a crowded trade area around Laizhou Bay coasts, with several fish piers and oil terminals located. Oil wastewater derived from tank washing water and fuel leakage of ships is inevitably discharged into the port area. The frequent activities of vessel transportation led to a relatively serious oil pollution in this area. Besides, wastewater from oil storages, petrol stations, dockyard, as well as other supporting facilities in the port is also possible oil pollution sources (He et al. 2018). The oil wastewater mentioned above may all contribute to the high concentration of TPHs in sediment from S5.

The sediment sample collected at S2 was identified as the maximal concentration of NP (26.98 μg kg^{-1}), followed by S3, S5, S7 and S8 (from 11.17 to 12.92 μg kg^{-1}), whereas the lowest values were from sites S1, S4, S6, S9, S10 and S11 (from 3.78 to 4.72 μg kg^{-1}). The highest concentration of NP in sediment collected at S2 may be related to the geographical position and water movement in this region. The average concentration of NP in the study area is 9.23 ± 0.41 μg kg^{-1}, which was in accordance with a previous study conducted by (Wang et al. 2010). In that study, an average concentration of 13.0 μg kg^{-1} NP was found in sampling sites along the Laizhou Bay coast.

Due to their lipophilic nature, organic pollutants in the coastal environment tend to accumulate in the soft tissues of organisms living in the benthic environment, both through contact with sediment or from feeding (Vigano et al. 2001). The contents of organic pollutants in the soft tissues of *R. philippinarum* are presented in Table 2. The sample from S10 showed the highest accumulation of total PCB concentration (26.69 μg kg^{-1}), followed by S2 (22.18 μg kg^{-1}) and S6 (20.12 μg kg^{-1}), while the lowest PCB content (4.22 μg kg^{-1}) was showed at site S5. Highly chlorinated biphenyls (PCB 101, 138, 153, 180, 194) counted for 53–89% of total PCBs in *R. philippinarum* at all biomonitoring sites (Fig. 2b). The highly chlorinated biphenyls can remain persistent in *R. philippinarum* because they are less volatile, more soluble in lipids, can be accumulated more readily to soft tissue of organisms, and are more resistant to biodegradation (Shiu and Mackay 1986; Tyler and Millward 1996). Levels of TPHs compounds were significantly higher in *R. philippinarum* from S3 (299.73 mg kg^{-1}) and S10 (300.72 mg kg^{-1}) compared with other sites (from 78.24 to 178.03 mg kg^{-1}). The highest accumulations of NP in *R. philippinarum* samples were found at S3 (165.53 μg kg^{-1}) and S9 (169.84 μg kg^{-1}), followed by S10 (126.35 μg kg^{-1}), whereas at the rest of the sites the concentrations were between 41.77 and 95.54 μg kg^{-1}. The average content of NP in *R. philippinarum* samples was 86.16 ± 5.29 μg kg^{-1}, which was lower than the data reported in Mussel (*Mytilus edulis*) and oyster (*Ostrea edulis*) samples collected in Laizhou Bay coastal areas (Wang et al. 2010). Species difference is the likely reason for the lower concentrations of NP found in the clams analyzed in this study. Besides, the concentrations of NP in the bivalves from Laizhou Bay were similar to those observed in Adriatic Sea (Italy), Bohai Bay (China) and Masan Bay (Korea) (Ferrara et al. 2001; Hu et al. 2005; Li et al. 2008).

Table 2
Concentrations of PCBs, TPHs, and NP in the surface sediments and *R. philippinarum* at eleven investigated stations (dry weight). Data are reported as mean ± standard deviation ($n = 3$). Different superscripted letters a, b, c, d, e and f indicate significant differences between sites. ($p < 0.05$)

site	sediments (dry weight)			*R. philippinarum* (dry weight)		
	∑PCBs ($\mu g\ kg^{-1}$)	TPHs ($mg\ kg^{-1}$)	NP ($\mu g\ kg^{-1}$)	∑PCBs ($\mu g\ kg^{-1}$)	TPHs ($mg\ kg^{-1}$)	NP ($\mu g\ kg^{-1}$)
S1	1.49 ± 0.09^{ab}	1.75 ± 0.19^{a}	4.57 ± 0.98^{a}	10.28 ± 0.74^{bc}	90.97 ± 1.34^{abe}	62.18 ± 6.49^{ab}
S2	1.22 ± 0.22^{ab}	22.74 ± 3.67^{ab}	26.98 ± 1.32^{c}	22.18 ± 3.62^{abcd}	173.56 ± 4.51^{de}	95.54 ± 11.21^{abc}
S3	1.01 ± 0.27^{ab}	13.83 ± 2.03^{ab}	11.43 ± 0.87^{b}	13.84 ± 1.11^{bcd}	299.73 ± 23.33^{f}	165.53 ± 24.41^{abc}
S4	6.56 ± 0.45^{c}	7.87 ± 1.42^{ac}	4.33 ± 0.25^{a}	14.09 ± 0.74^{cd}	78.24 ± 4.53^{a}	44.56 ± 2.29^{a}
S5	0.68 ± 0.05^{a}	285.96 ± 16.69^{d}	11.17 ± 1.23^{b}	4.23 ± 0.42^{a}	142.69 ± 8.41^{cd}	41.77 ± 3.54^{a}
S6	1.25 ± 0.12^{ab}	35.45 ± 5.56^{bc}	3.95 ± 0.15^{a}	20.14 ± 1.67^{d}	161.23 ± 5.92^{bcd}	49.12 ± 2.34^{a}
S7	0.74 ± 0.06^{a}	6.04 ± 0.75^{ac}	12.89 ± 0.64^{b}	12.15 ± 0.87^{bc}	119.38 ± 11.19^{abcd}	83.35 ± 5.53^{bc}
S8	1.93 ± 0.07^{b}	9.52 ± 2.14^{ac}	12.92 ± 1.27^{b}	9.72 ± 0.97^{b}	129.59 ± 9.68^{abc}	48.64 ± 3.38^{a}
S9	0.72 ± 0.03^{a}	29.56 ± 6.65^{ab}	4.75 ± 0.54^{a}	19.16 ± 2.46^{abcd}	178.03 ± 9.43^{bcd}	169.84 ± 15.32^{c}
S10	3.53 ± 0.22^{c}	19.14 ± 1.41^{b}	3.78 ± 0.35^{a}	26.69 ± 4.23^{abcd}	300.72 ± 10.25^{f}	126.35 ± 16.67^{abc}
S11	1.72 ± 0.21^{ab}	3.23 ± 0.42^{a}	4.72 ± 0.68^{a}	10.45 ± 1.26^{bc}	150.12 ± 1.26^{cd}	60.93 ± 4.35^{ab}
Mean ± SD	1.90 ± 0.10	39.55 ± 2.42	9.23 ± 0.41	14.81 ± 0.96	165.87 ± 5.03	86.16 ± 5.29

Linear regression analysis on the levels of organic pollutants accumulated in *R. philippinarum* and in sediments showed no significant correlations in this study. The accumulation of pollutants in biota is a complex process that does not only depend on their concentration in the environment. Environmental physicochemical factors including temperature, salty, DO and nutritional status can influence the

organism conditions, which may cause further effects on the bioavailability of organic pollutants. In this study, there were no evident changes in the physical-chemical parameters of water at different sites along Laizhou Bay apart from DO, which showed the lowest content at S1(5.36 mg L^{-1}), as shown in Table 1. Coincidentally, the lowest condition index value of clams was also found in *R. philippinarum* collected from S1, indicating that the lack of DO would cause negative impacts on the growth and health status of *R. philippinarum*. Nevertheless, neither Pearson nor Spearman correlation analysis showed a significant correlation between DO and the condition index of *R. philippinarum*. Moreover, no significant correlation relationships were found between non-pollution factors (temperature, salty, DO) and pollutants concentration in *R. philippinarum* tissues. Therefore, we deduce that the effects of a single environmental factor were inconspicuous on pollutants accumulation in organisms, and all the environmental parameters may have a comprehensive effect on the process of pollutants accumulation. Furthermore, several factors may also be responsible for the poor correlation between the concentration of pollutants in sediments and organisms, including the total organic carbon content of sediments, estuary deposition, characteristics of the water body, harbor activity and differences in the ecosystem characteristics of the coastal environment (Wang et al. 2010).

Biomarker responses in gills and the digestive glands of R. philippinarum

GSHt content, SOD, CAT, GPx and GST activities and LPO level in the gills and digestive glands of *R. philippinarum* collected from eleven stations are presented in Fig. 3. Differences of biomarker responses between gills and digestive glands were showed in each of the measured bioindicators. In all of the investigated stations, *R. philippinarum* presented higher SOD, GST, GPx and GSHt levels in gill tissues compared with digestive gland. Gill tissue in bivalves has a wide surface area which initially and continuously contacts the external medium, and is considered as the main uptake route of contaminants. Higher levels of antioxidants (SOD, GST, GPx and GSHt) in gills implied an adaption to the environmental stress, protecting the gill tissue from oxidative damage caused by pollutants as well as their harmful metabolites. Nevertheless, LPO levels was not markedly different between the two tissues.

Significant differences between sites were also observed in most biomarker responses in gills. Higher activities of SOD, GST and GPx were found simultaneously at S5, S7 and S8 whereas a relatively lower LPO level was observed, while lower activities of SOD, GST and GPx were observed in *R. philippinarum* collected from S2, which presented a higher LPO level compared with all other sites. The similar response pattern of SOD, GST and GPx indicated that these enzymes operate together in the process of scavenging ROS. However, LPO is considered as a biomarker of oxidative damage, which usually makes it exhibit contradictory trends against the antioxidant enzymes (Wang and Cui 2016). The biomarker responses in digestive glands did not differ much among stations except for CAT, the activity of which was higher at S8 and S9. Generally, biomarker responses in gills of *R. philippinarum* exhibited more conspicuous diversities among stations, indicating a sensitive reaction to environmental changes.

Correlations Between Parameters

Relationships among biomarkers

Overall, oxidative biomarkers exhibit functions coordinated with each other so as to protect the living organisms from damage. In this study, we have identified several relationships between different biomarkers by using the methods of Pearson correlation analysis as well as Spearman correlation analysis. Compared with Spearman correlation analysis, results of Pearson correlation analysis were preferable to explain the relationships between biomarkers and thus were adopted in the discussion. Non-significant correlations were not presented.

The results of Pearson correlation analysis showed a significant positive correlation between SOD_g and GPx_g ($r = 0.91$, $p < 0.01$) in *R. philippinarum*, as well as between SOD_{dg} and CAT_{dg} ($r = 0.62$, $p < 0.05$) (subscripts g and dg representing biomarkers in gills and digestive glands, respectively). SOD, CAT and GPx are all antioxidant enzymes which function together in the process of ROS clearance. SOD catalyzes the transformation of superoxide radicals to H_2O_2, which is subsequently degraded into H_2O by CAT and GPx (Wang and Cui 2016). SOD and CAT act as important frontiers for defending against ROS toxicity (Wang et al. 2011). Previous studies have proved that CAT is the first enzymatic defense against H_2O_2 in the kidneys of *Liza aurata* (Oliveira et al. 2010), while other studies reported that GPx is easily increased in response to oxidative stress (Basha and Rani 2003). Moreover, it has been reported that in the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPx; however, the organic peroxides are mainly metabolized by CAT at high H_2O_2 concentrations (Yu 1994). Relationships observed in this study indicated that H_2O_2 which generated in the process of SOD catalysis was degraded mainly by CAT in the digestive glands of *R. philippinarum*, while GPx showed the same function as CAT in gill tissues. The explanation corresponds well to the tissue distribution regularities of these enzymes.

GST plays an important role in catalyzing the conjugation of the tripeptide glutathione with the xenobiotic in phase □ of the biotransformation process and promotes its elimination from the organism (Richardson et al. 2008). In this study, linear relationships were found between GST_g and SOD_g ($r = 0.749$, $p < 0.01$), CAT_g ($r = 0.750$, $p < 0.01$), GPx_g ($r = 0.797$, $p < 0.01$), as well as GST_{dg} with SOD_{dg} ($r = 0.654$, $p < 0.05$), CAT_{dg} ($r = 0.632$, $p < 0.05$). The results was in accordance with those obtained by Qiu et al. (2007) for *Hypophthalmichthys molitrix*, where GST activities were also positively associated with SOD, CAT and GPx. Although the specific mechanisms for this result remain unclear, we deduce that GST can function similarly to GPx in the organisms during ROS-scavenging process.

Relationships between biomarkers and non-pollution parameters

Previous studies have proved that water physical-chemical parameters such as temperature, salinity, pH and DO have impacts on biomarker responses (Menezes et al. 2006; Ringwood and Keppler 2002; Santovito et al. 2005), therefore it is necessary to avoid the disturbances of these factors as far as possible in biomonitoring programs. In this study, as the physicochemical features of seawater (temperature, salinity and pH etc.) were similar at the sampling sites of Laizhou Bay, it could be implied that the biomarker responses of the clams were not disturbed by these environmental factors excessively.

However, DO content of the seawater from S1 was significantly lower than other sampling sites. Santovito et al. (2005) studied the biomarker response features of mussel *Mytilus galloprovincialis* at two sites with different DO levels in Lagoon of Venice and found that antioxidant enzyme activity had a closely correlation with water DO. In this study, no significant correlations between DO and the biomarkers were found, which implied that the difference of DO level among different sites had no obvious impacts on the biomarker responses of *R. philippinarum*. Apart from the abiotic factors mentioned above, biotic factors (virus, bacteria, parasite etc.) also have possible influences on biomarkers. Minguez et al. (2012) studied the parasite infra-communities and zebra mussel biological responses up- and downstream a waste water treatment plant in northeast France and found that the biological responses differences were observed both related to the site environmental quality and the infection status induced by ciliates and intracellular bacteria. Nevertheless, as parasitism in the collected organisms were unknown in our research, the possible influences of parasite on biomarker responses of *R. philippinarum* remained unclear and deserves further investigations.

Relationships between biomarkers and organic pollutants

Pearson correlation analysis was used to obtain the relationships between biomarkers and organic pollutants. The significant correlations obtained between biomarker responses and organic pollutants in *R. philippinarum* are presented in **Fig. 4 (a)**. No dependences were found between biomarker responses and pollutants' concentrations in sediments. Many field studies employing bivalves showed increased GST and GPx activities in organisms collected from polluted sites (Cheung et al. 2002). However, in the present study, GPx in gills decreased linearly with accumulated PCBs and NP content in *R. philippinarum*, while GST in gills was only significantly correlated with PCBs. In general, under long-lasting contamination conditions, excess toxicity of accumulated oxidizing agents resulted in enzyme inhibition, exhibiting oxidative damages in organisms. Therefore, the decreased GST and GPx activities in organisms may be due to the long-lasting organic pollution in the study area.

In addition to the antioxidant enzymes, reduced glutathione (GSH) in organisms also plays an important role in ROS-scavenging process (Pandey et al. 2003). Generally, GSH is likely to be oxidized into oxidized glutathione (GSSG) when the organism is under oxidative pressure, resulting in an elevated GSSG content in organism tissues. In this study, GSHt content in the digestive glands was positively correlated with NP accumulation in *R. philippinarum*. The increase in GSHt content reflects an adaptation to pollution pressure and has been shown to play a critical role in maintaining cellular homeostasis (Doyotte et al. 1997).

To exclude the possible bias induced by outliers, Spearman correlation analysis was also conducted to assess the relationship between biomarker responses and organic pollutants, results of which were presented in **Fig. 4 (b)**. The results showed that new linear relationships were found between biomarker responses and organic pollutants in *R. philippinarum*. SOD in gills decreased linearly with accumulated TPHs and NP content in *R. philippinarum*, while GPx in gills was significantly correlated with TPHs. According to the agreement of results obtained from Pearson correlation analysis and Spearman correlation analysis, as can be seen from **Fig. 4**, it could be inferred that the organic pollutants

accumulated in *R. philippinarum* tissues inhibited the activities of enzymes (SOD, GPx and GST) on the whole. Moreover, the biomarkers in the gills of *R. philippinarum* responded more sensitively to xenobiotics compared to those in the digestive glands.

Calculation of IBR

IBR has been previously used as a tool for assessing environmental risk and for a general description of the “health status” of populations combining the different biomarker signals. Biomarkers in different organism organs respond variably to external or internal changes because each of these tissues plays a specific role in organism protection (Pereira et al. 2010a). In previous studies which applied the IBR approach, biomarkers employed to calculate IBR values were mostly from a single tissue (gill, digestive gland, liver, etc.) of the studied organisms (Jebali et al. 2011; Oliveira et al. 2009; Pereira et al. 2010b; Wang et al. 2011). In general, the tissue that subtly respond to environmental changes is favorable, and a battery of biomarkers from the same tissue is recommended for IBR calculation in biomonitoring research (Meng et al. 2012). In the present study, biomarkers from two different tissues in the clam were determined, and thus a procedure for biomarker selection was necessary before the IBR calculation.

In our study, it has been proved that biomarkers in the gills of *R. philippinarum* showed higher expression and responded sensitively to organic pollutants, and thus were selected to calculate the IBR index. The IBR values were depicted by star plot (**Fig. 5**). In general, IBR values showed a large range of variation among different monitoring sites. In consideration of the extent and shape of the star plot, it was clear that S8 (0.02) near Furong Island and S7 (0.05) near Hutouya, a small fishing village, were the less impacted sites, whereas S2 (IBR = 2.65) was identified as the most affected site. The rank of all the stations could be ordered as: S2 (2.65) > S9 (1.26) > S4 (1.13) > S3 (1.08) > S11 (0.89) > S1 (0.74) > S10 (0.69) > S5 (0.58) > S6 (0.18) > S7 (0.05) > S8(0.02). Overall, the western coast (S1-S4) and eastern coast (S9-S11) exhibited higher environmental stress than the sampling sites along the southern coast of Laizhou Bay, which can be attributed to the combined effect of tidal currents, sediment characteristics and human activities (Liu et al. 2017; Zhang et al. 2017).

The IBR index was regarded as a practical tool which could be applied to examine the stress response of different populations by the combination of different biomarkers. Furthermore, it can provide an effective comparison between IBR values and pollutants, which can point out the main influencing pollutants of the study area. In this study, significant Spearman correlation coefficients were found between IBR and PCBs ($r = 0.627$, $p<0.05$), NP ($r = 0.664$, $p<0.05$) in *R. philippinarum*, which indicated that PCBs and NP have caused high level of pollution pressure on *R. philippinarum* in sediment environment along Laizhou Bay coastal area.

Conclusions

A field study was conducted to evaluate the environmental conditions and stresses caused by toxic organic pollutants around the coastal area of Laizhou Bay by using a biomonitoring method combined with chemical analysis. In summary, pollution of PCBs, TPHs and NP were present in the whole range and have caused different degrees of environmental stresses in all the investigated stations, as indicated by the native bivalve *R. philippinarum*. According to the results of biomarker responses, gill tissue of *R. philippinarum* demonstrated its usefulness in biomonitoring studies. IBR values exhibited noticeable spatial variation in our research and S2 showed the highest IBR value, indicating a more stressful contamination for *R. philippinarum* than other areas. The western coast (S1-S4) and eastern coast (S9-S11) exhibited higher environmental stress than the sampling sites along the southern coast of Laizhou Bay. PCBs and NP were the main organic pollutants that have caused high level of pollution pressure on *R. philippinarum* in Laizhou Bay coastal area. In conclusion, the biomonitoring method combined with IBR analysis was proved to be useful for discrimination between different pollution sites. However, no biomarkers were found to fully respond to TPHs compounds in our multi-biomarker study. As different biomarkers respond to different stressors, more kinds of biomarkers should be measured along with those already adopted in this study, and the seasonal variations of IBR levels related to pollutant concentrations in sediments and organisms should be carried out in the future.

Declarations

Authors' contributions

ZW conceived and designed the study, performed the chemical and biomarker analysis, data analysis, and manuscript preparation. YS, CL, LL and LZ provided support for sampling, chemical and biomarker analysis. DW supervised the draft. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

For this study using bivalve, formal consent is not required.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Figures

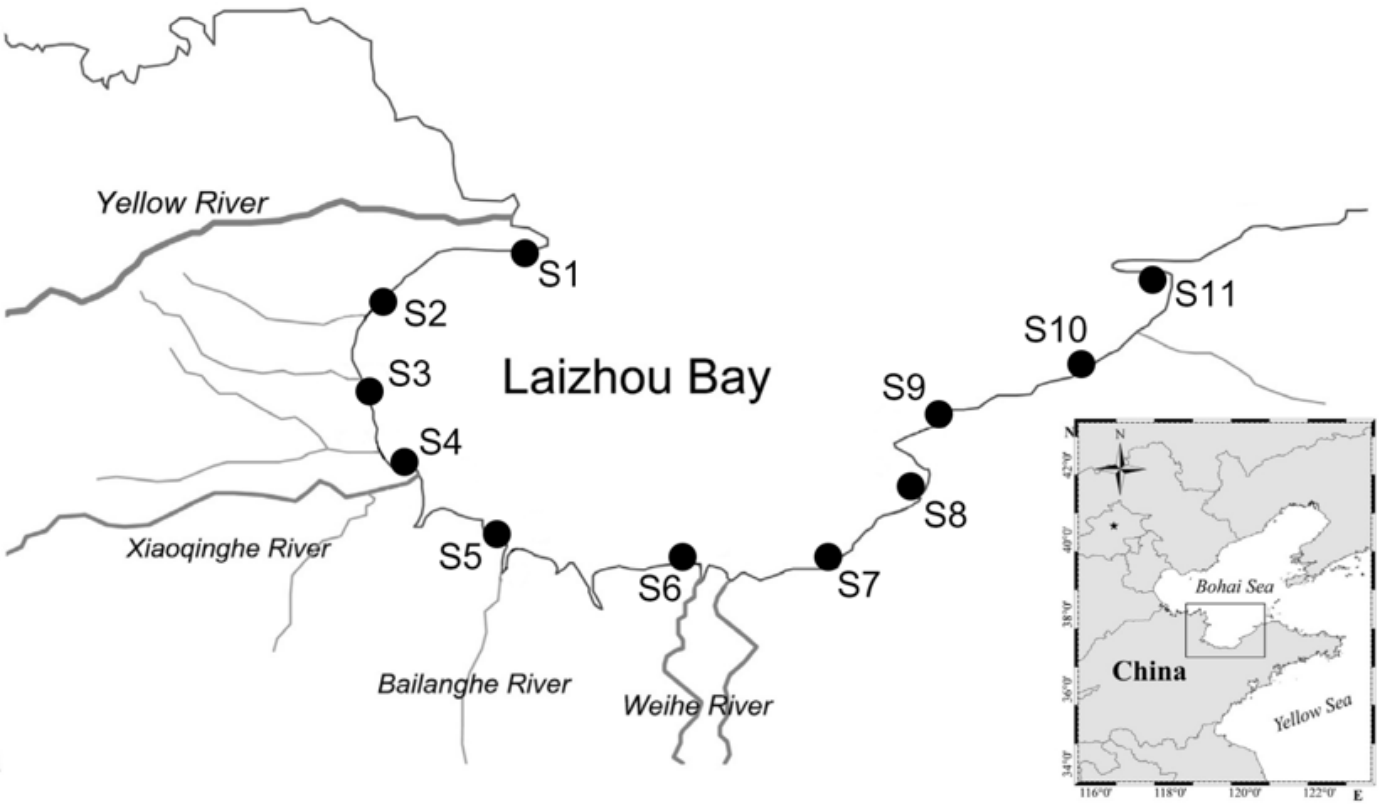

Figure 1

Location map of the sampling stations along the coast of Laizhou Bay, China: S1 (E119°07'44.29" N37°43'34.88"), S2 (E118°57'50.41" N37°36'04.37"), S3 (E118°54'57.37" N37°30'3.15"), S4 (E118°59'18.26" N37°17'46.71"), S5 (E119°12'16.30" N37°11'33.45"), S6 (E119°29'46.13" N37°09'14.98"), S7 (E119°45'46.06" N37°09'38.04"), S8 (E119°52'55.17" N37°16'50.20"), S9 (E119°56'6.08" N37°24'36.06"), S10 (E120°12'17.74" N37°31'21.19") and S11 (E120°16'47.59" N37°40'1.01").

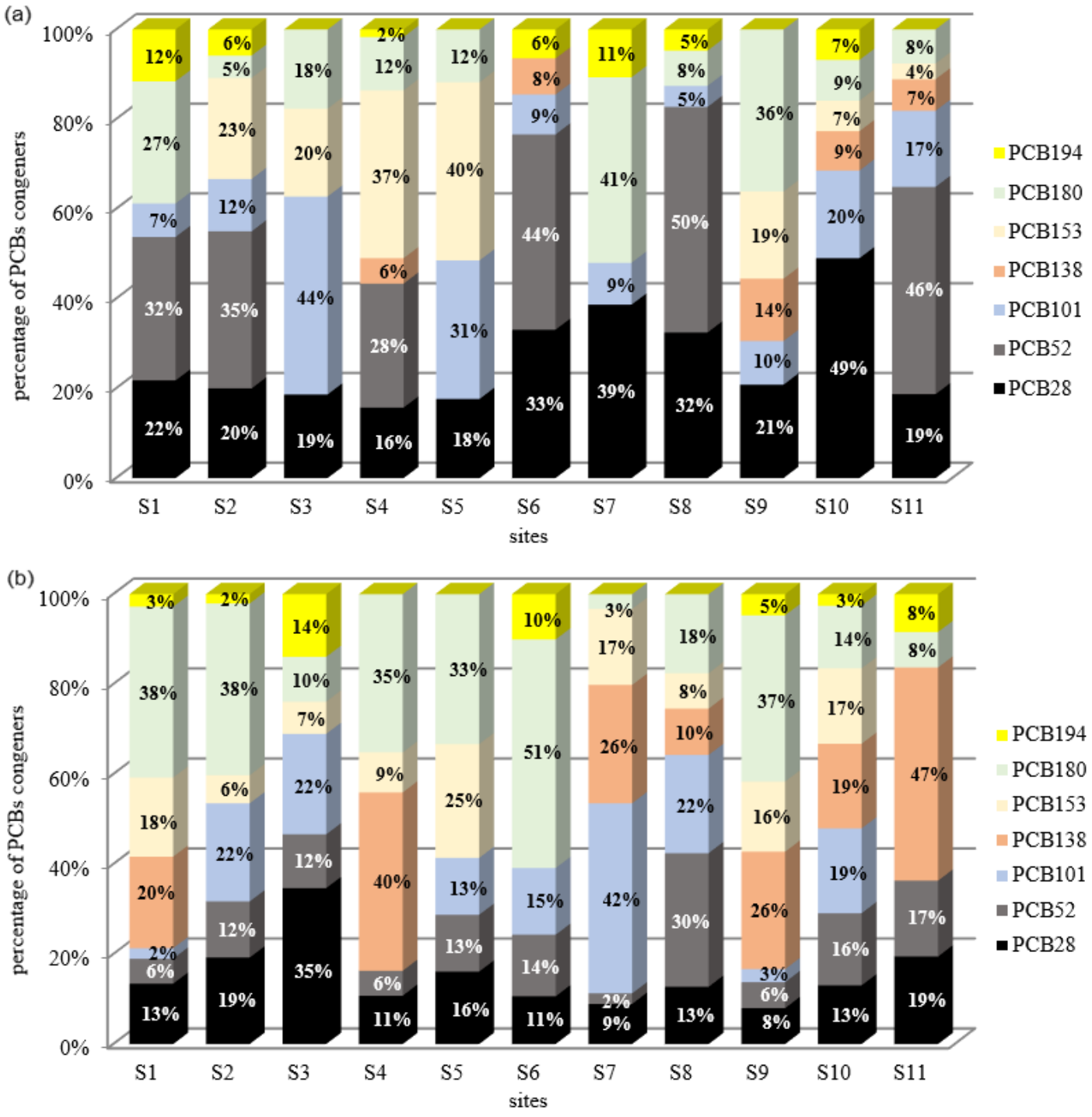

Figure 2

Percentage of PCBs congeners in sediments (a) and R. philippinarum (b) collected from eleven investigated stations.

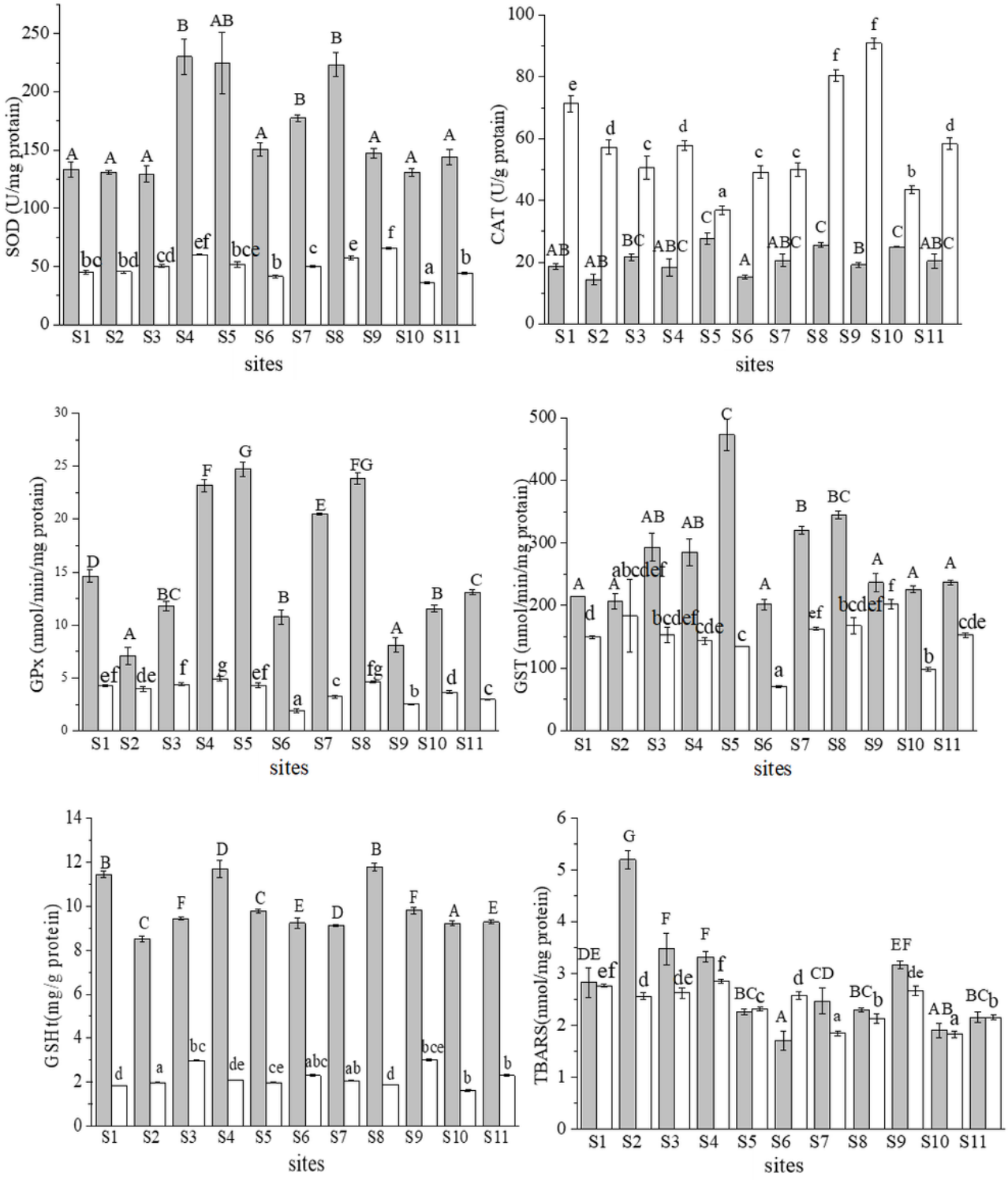

Figure 3

Biomarker responses measured in gills (grey columns) and digestive glands (white columns) of R. philippinarum collected from eleven sites. All results are expressed as mean ± SD (n = 3). Letters A, B, C, D, E, F and G (or a, b, c, d, e, f and g) indicate a significant difference between sites. ($p < 0.05$)

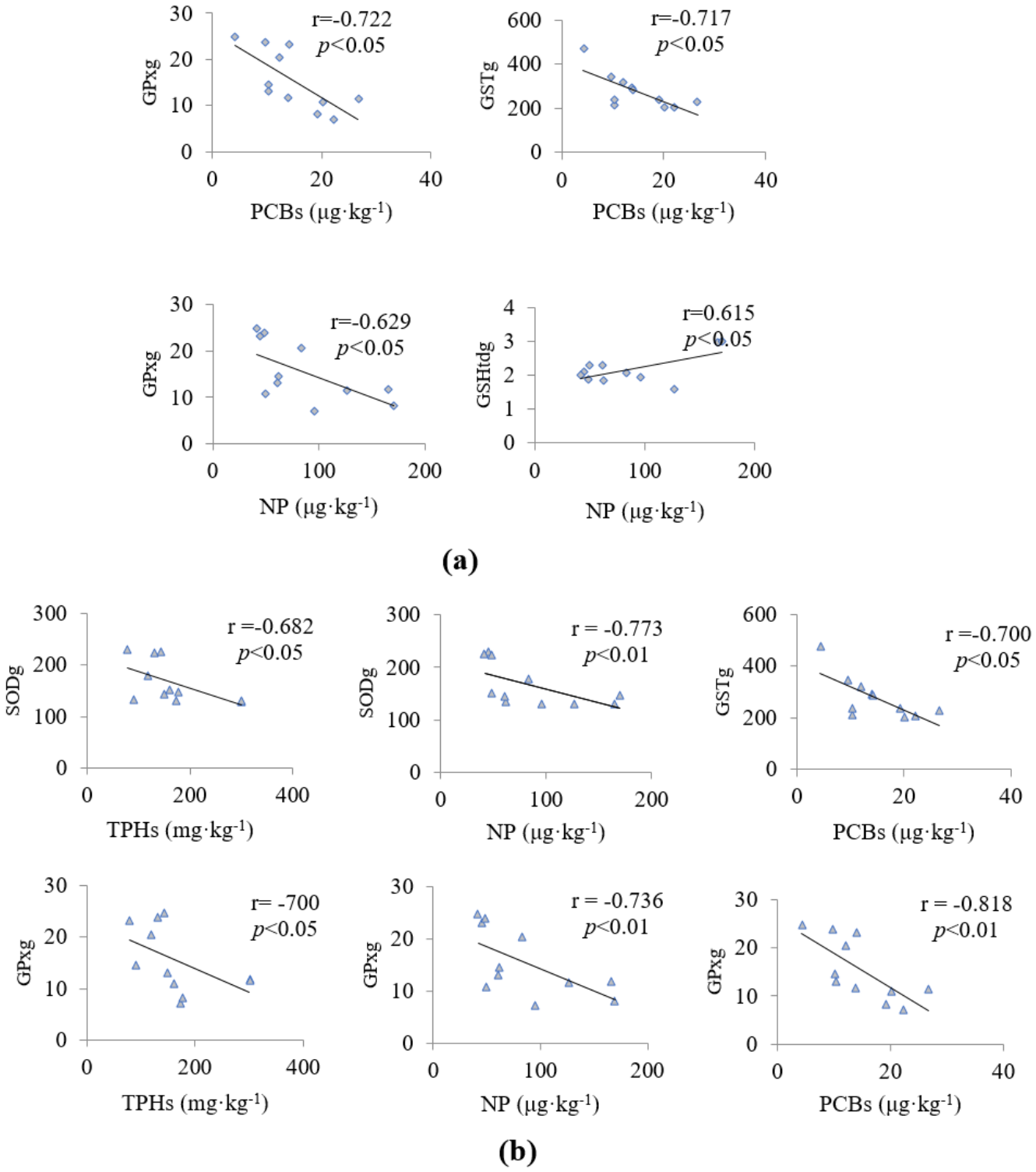

Figure 4

Significant correlations of biomarker responses with organic pollutants levels in R. philippinarum. Statistical significance and correlation coefficient are represented by p and r. Subscripts g and dg represent gill and digestive gland, respectively. (a-Pearson correlation analysis, b-Spearman correlation analysis)

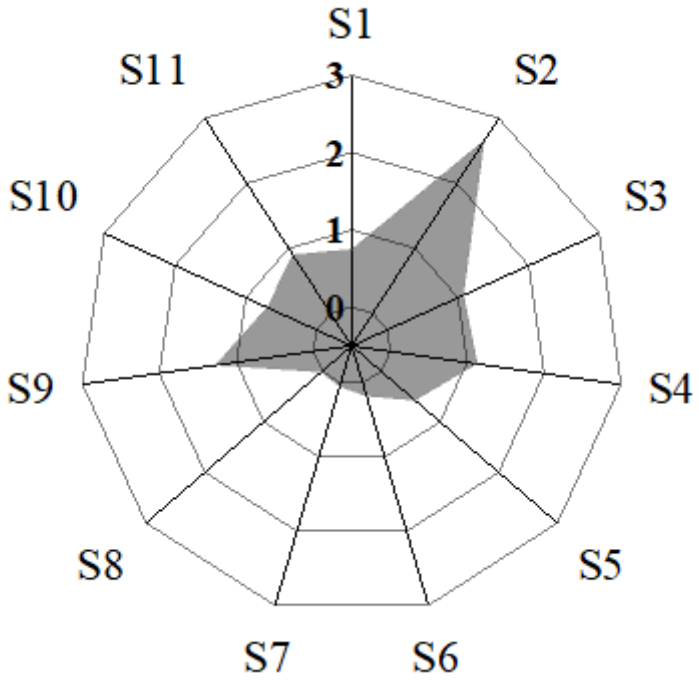

Figure 5

Star plot for integrated biomarker response (IBR) values of the eleven investigated stations.