



Addressing the challenge of biomonitoring without reference site: strategies from the Jiulong River estuary and nearby coastal seas, China*

Ronghui ZHENG¹, Chao FANG¹, Yu WANG², Fukun HONG¹, Fulong GAO¹,
Yusheng ZHANG¹, Jun BO^{1,**}

¹ Key Laboratory of Marine Ecological Conservation and Restoration, Third Institute of Oceanography, Ministry of Natural Resources, Xiamen 361005, China

² Laboratory of Marine Biodiversity, Third Institute of Oceanography, Ministry of Natural Resources, Xiamen 361005, China

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Abstract Environmental variability (e.g., temperature, salinity, pH) and seasonal fluctuations in pollutant concentrations often hinder the establishment of reliable reference sites in marine biomonitoring, challenging the accurate assessment of pollutant-induced biological effects. This study employed tiered biomonitoring and assessment approaches to evaluate endocrine disrupting compounds (EDCs) using embryos of *Oryzias melastigma* in the Jiulong River estuary (JRE) and nearby coastal seas of China, that are characterized by significant environmental variability and distinct seasonal fluctuations in pollutant concentrations. The findings demonstrated that the levels of alkylphenols and bisphenol A in the JRE and nearby coastal seas are relatively low to moderate in contrast to those found in other regions of the world. Consistent evidence from the chemical analyses, expression patterns of five EDC-inducible genes, and both integrated biomarker response (IBR) and integrated biomarker response version 2 (IBRv2) tiered-assessment systems identified sites S10 and S11 (Xiamen Western Harbor) as exhibiting the most severe EDC pollution. Correlation analysis confirmed the approximate functional equivalence between IBR and tiered-assessment approaches. It also demonstrated that IBR tiered-assessment approach provides a robust tool for biomonitoring in marine environments where establishing reference site is challenging.

Keyword: biomonitoring; estrogenic endocrine disrupting compound; embryotoxicity; integrated biomarker response; tiered assessment

1 INTRODUCTION

In environmental monitoring, chemical methods are effective for detecting specific types, concentrations, and distributions of pollutants. However, they are generally restricted to preselected contaminants and cannot comprehensively assess all pollutants present in the environment. Moreover, they do not directly reflect their actual effects on organisms (Geiszinger et al., 2009). In response to these constraints, biological approaches, more commonly known as biomonitoring, have been developed as a complementary strategy.

Biomarkers, widely employed in biomonitoring, serve as ecotoxicologically valuable early-warning

indicators. They enhance understanding of cause-effect relationships and provide insights into the biological effects of pollutants, contributing to the assessment of environmental stress (Yang et al., 2023; Rabuffetti et al., 2024). To ensure reliable assessment of aquatic pollutant impacts and minimize misinterpretation in complex pollution scenarios, careful selection of biomarker batteries is essential (Ferreira et al., 2005). For instance, biomarker batteries cytochrome P450 aromatase

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** Corresponding author: bojun@tio.org.cn

(CYP19), choriogenin (Chg), testosterone (T), estrogen (E2) and vitellogenin (Vtg) have been successfully employed to assess the impact of endocrine disrupting compounds (EDCs) in fish (Solé et al., 2016; Ye et al., 2018; Zhang et al., 2020).

Field applications of biomarkers, however, are often confounded by abiotic factors (i.e., physicochemical parameters) (Slaby et al., 2024). To address this, the integrated biomarker response (IBR) method synthesizes multi-biomarker data into a single stress index via star plots, enabling site-to-site environmental stress comparisons (Beliaeff and Burgeot, 2002). Its refinement, integrated biomarker response version 2 (IBRv2), further quantifies pollution severity and facilitates cross-site comparisons (Sanchez et al., 2013). Both methods are extensively applied in field research (Fang et al., 2020; Yang et al., 2023). Crucially, these indices translate complex biological responses into actionable metrics while systematically classifying ecological impacts.

To address the research gap in field-based, tiered assessment of pollutant effects, we have developed a novel tiered-assessment approach by modifications of the expert systems (Dagnino et al., 2007) and IBRv2 (Sanchez et al., 2013). This approach was successfully implemented to rank the biological effects of EDCs on *Sebastiscus marmoratus* in a field study that utilized caged organisms and reference sites (Zheng et al., 2021). However, that the effectiveness of such controlled designs in broader biomonitoring can be limited by environmental variability (e.g., temperature, salinity, pH) (Martinez-Haro et al., 2022; Catteau et al., 2023; Slaby et al., 2024) and seasonal pollutant fluctuations (Zhang et al., 2019; Peluso et al., 2021; Carreras-Colom et al., 2022) often preclude reference site establishment in biomonitoring. In such scenarios requiring tiered assessment of pollution-induced biological effects, the IBR approach remains applicable, whereas IBRv2 cannot be implemented due to its dependence on reference sites.

Therefore, this study aimed to evaluate the applicability of the IBR methodology for tiered biological effect assessment, benchmarked against our established IBRv2 framework (Zheng et al., 2024). In the Jiulong River estuary (JRE) and nearby coastal seas of China, twelve sampling stations were selected, characterized by significant environmental variability and seasonal pollutant

fluctuations. Using the marine model fish *Oryzias melastigma*, we quantified the impacts of early-life exposure to surface seawater, investigated biological responses across subcellular to organismal levels, and evaluated contextual applicability of IBR- versus IBRv2-based tiered-assessment approaches.

2 MATERIAL AND METHOD

2.1 Study area and sampling

As the second longest river in Fujian Province, southeastern China, the Jiulong River traverses three major cities (Longyan, Zhangzhou, and Xiamen) before draining into Xiamen Harbor (Liu et al., 2006). The riverine runoff entering the JRE from this system averages 14.8 billion m³ per year (Chen et al., 2014). The Jiulong River has been impacted in recent years by both extraordinary climate change (e.g., drought and deluge) and extensive anthropogenic activity (e.g., dam impoundment of cascade hydropower projects, agricultural and industrial wastewater) (Chen et al., 2015; Wang et al., 2022). As a consequence, the pollution load of the JRE has been gradually increased. Furthermore, the Xiamen Economic Special Zone's explosive economic growth since 1986 has caused severe anthropogenic disruption and substantial environmental stress for the JRE and its surrounding regions (Wu et al., 2016).

Sampling was conducted at twelve sites in the JRE and nearby coastal seas in October 10–11, 2021 and July 15–16, 2022 (Fig.1; Supplementary Table S1). Surface seawater (0.5-m depth) was sampled for chemical analysis with pre-cleaned brown glass bottles at each site. The 10-L opaque, food-grade plastic buckets devoid of bisphenol A were used to store the surface seawater samples for embryonic exposure. Every sample was gathered, processed, and shipped in compliance with national criteria (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China and Standardization Administration of the People's Republic of China, 2008).

2.2 Chemical analysis

In accordance with the “Code of Practice for Marine Monitoring Technology Part 1: seawater” (State Oceanic Administration, People's Republic of China, 2013), the concentrations of three representative estrogenic EDCs in surface seawaters: two alkylphenols (APs, 4-nonylphenol (4-NP) and

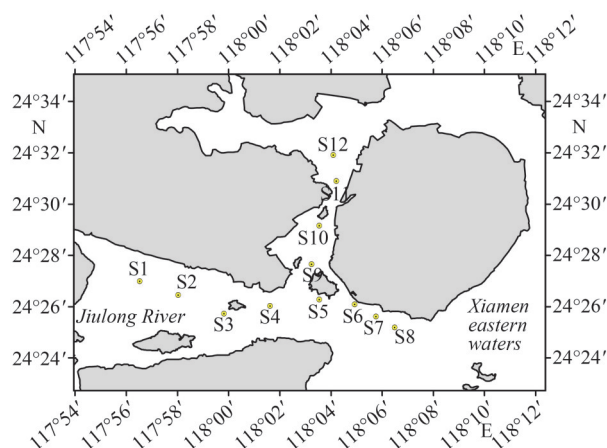


Fig.1 Study area and sampling sites

4-*tert*-octylphenol (4-*t*-OP)) and bisphenol A (BPA), were measured using gas chromatography-mass spectrometry (GC-MS) (Kang et al., 2002; Watanabe et al., 2017; Maciuszek et al., 2020). A SH-Rxi-5ms (30 m×0.25 mm) chromatographic column with a particle size of 2.5 µm was utilized (Shimadzu Co. Ltd., Japan). Using internal standards, the mean recoveries of APs ranged from 78.2% to 96.7%. For 4-NP, 4-*t*-OP, and BPA, the corresponding detection limits (extraction volume: 1 000 mL) were 0.006, 0.002, and 0.010 ng/L, respectively.

2.3 Maintenance of experimental fish

The marine model fish *O. melastigma* were acquired from the City University of Hong Kong, and large-scale culturing was performed following standard operating procedures (Bo et al., 2011). Fish embryos were prepared following the protocol described by Zhang et al. (2021). Briefly, the procedure included: (i) assessing egg health under a stereomicroscope (Nikon SMZ1270; Nikon Corp., Japan) prior to the experiment; (ii) randomizing fertilized eggs into 20-cm diameter glass petri dishes for incubation in 30% aerated artificial seawater at 28 °C; and (iii) selecting healthy embryos that had been cultured for 48 h post-fertilization for the subsequent exposure. The study was approved by the ethics committee of Third Institute of Oceanography, Ministry of Natural Resources, China (approval No. TIO-IACUC-01-2024-03-08).

2.4 Embryonic exposure and sampling

Oryzias melastigma embryos were exposed from 3 to 6 days post-fertilization (dpf), for a total

exposure duration of 96 h, which covered two essential early-life stages: 3 dpf, characterized by full heart development, and 6 dpf, by which time the liver is well formed (Chen et al., 2020).

For each exposure condition (i.e., seawater from each of the 12 sampling sites and the laboratory control), five biological replicates were established. Each biological replicate consisted of a 12-cm diameter glass Petri dish containing 100 mL of the test solution and 20 randomly allocated healthy embryos. Two-thirds of the test solution in each dish was renewed daily with the corresponding seawater sample.

After 96 h of exposure, pooled samples comprising 15 embryos from each replicate were flash-frozen in liquid nitrogen and subsequently stored at -80 °C. The preserved samples were destined for transcriptional expression analysis using real-time quantitative PCR (RT-qPCR). Throughout the exposure, deceased embryos were removed and recorded promptly. Embryonic heart rate and the incidence of malformations were documented daily.

2.5 Teratogenicity

Following the 96-h exposure, the teratogenicity was assessed for all surviving embryos in each replicate according to established protocols by evaluating the presence of blue sac disease (BSD) (Zheng et al., 2024). Briefly, each embryo was evaluated and scored according to the following criteria: a score of “0” indicated no abnormalities; a score of “1” indicated the presence of one or more mild to moderate malformations, such as abnormal heart rate, cardiac defects, reduced activity, spinal or craniofacial deformities, fin erosion, or localized hemorrhaging; and a score of “3” indicated severe signs strongly associated with mortality, including yolk sac edema or pericardial edema. For each group, the final BSD score was determined by averaging the total scores of all embryos for each toxicity sign.

The heart rate was measured as a separate endpoint. For each exposure condition, 10 embryos were randomly selected from across the five biological replicates. The heart rate (beats per minute, bpm) for each embryo was calculated by timing 30 heartbeats using the formula: $N = (30/T) \times 60$, where T is the elapsed time in seconds.

2.6 Real-time quantitative PCR assay

The expression levels of five genes—aromatases

cyp19a and *cyp19b*), choriogenin (*chgL* and *chgH*), and vitellogenin (*vtg*)—which have been established as convenient biomarkers for evaluating the effects of estrogenic EDCs, were quantified using RT-qPCR (Fang et al., 2012; He et al., 2012). 18S ribosomal RNA (18S rRNA) served as the endogenous control (Bo et al., 2011). The primer sequences employed in this study are provided in Supplementary Table S2. RT-qPCR was conducted on a fluorescent quantitative thermal cycler (Rotor-Gene Q 5ples; Qiagen, Germany). Data analysis was performed following the method established by Bo et al. (2011).

2.7 Tiered assessment of the biological effects

The biological effect levels were classified into five tiers based on a tiered-assessment framework (Zheng et al., 2024), which examines effects across molecular/cellular, tissue, and organism levels. The classification criteria were as follows: Level V (survival stress) was assigned when a treatment group exhibited a statistically significant decrease in survival rate at the organism level. Level IV (high stress) was assigned in the absence of organism-level effects but with significant increase in the BSD score at the tissue level. When adverse effects were observed only at the molecular/cellular Level, the stress level was assigned as I (no stress), II (low stress), or III (medium stress) by comparing the IBR or IBRv2 values against established thresholds (sections 2.7.1 and 2.7.2).

2.7.1 IBR and IBRv2 calculation

To avoid the order bias, the IBR value was calculated based on the method of Devin et al. (2014) through the following steps: (i) for each biomarker, calculate the mean (X_i), general mean (m_i), and standard deviation (s_i); (ii) standardize the value: $Y_i = (X_i - m_i) / s_i$; (iii) adjust for response direction: $Z_i = Y_i$ (if increasing) or $Z_i = -Y_i$ (if decreasing); (iv) compute the score: $S_i = Z_i + |\min_i|$ where $|\min_i|$ is the absolute minimum Z_i ; (v) calculate the area between two biomarkers on the star plot: $A_i = \frac{1}{2} S_i \times S_{i+1} \times \sin\left(\frac{2\pi}{n}\right)$; (vi) sum all areas to obtain the IBR: $\text{IBR} = \sum_{i=1}^n A_i$. The final IBR value was the average of multiple calculations performed with randomized orders of biomarkers to eliminate ordering effects.

The IBRv2 was computed using the following procedure described by Sanchez et al. (2013): (i) for each biomarker, the ratio of its value in the treatment group (X_i) to that in the control group (X_0)

was log-transformed to reduce variance: $Y_i = \lg(X_i / X_0)$; (ii) the values were standardized using the overall mean (μ) and standard deviation (s): $Z_i = (Y_i - \mu) / s$; (iii) the difference from the reference value (Z_0) was determined: $A = Z_i - Z_0$; and (iv) the IBRv2 value was obtained by summing the absolute deviations: $\text{IBRv2} = \sum |A_i|$.

2.7.2 Determination of the graduated thresholds

Each biomarker was assigned an alteration Level (no, low, or moderate) by applying threshold values to the IBR (minimum value) or IBRv2 (control) results, adapting a method from Dagnino et al. (2007). For biomarkers with a decreasing response, the thresholds were 1.0, 0.8, and 0.5; otherwise, the thresholds were 1.0, 1.2, and 2.0. These thresholds were used to establish graduated levels within the IBR/IBRv2 algorithm.

2.8 Statistical analysis

All data are expressed as mean $\pm$ standard error (SE) and were analyzed using SPSS software (v26.0; SPSS Software Inc., USA). Given the non-normality of the data, the non-parametric Kruskal-Wallis test was employed. Correlations between IBR and IBRv2 values were examined using Spearman's rank correlation analysis. Statistical significance was set at $P < 0.05$, and all graphs were generated using GraphPad Prism (v8.4; GraphPad Software Inc., USA).

3 RESULT

3.1 Concentration of chemicals in surface seawater

The concentrations of 4-NP, 4-*t*-OP, and BPA are detailed in Table 1. In surface seawater, 4-NP was below the detection limit (n.d.) in all samples from October 2021, while in July 2022, its concentrations varied from n.d. to 8.35 ng/L. 4-*t*-OP concentrations ranged from 4.51 to 27.96 ng/L (October 2021) and 5.57 to 13.29 ng/L (July 2022). BPA concentrations varied from n.d. to 21.56 ng/L in October 2021 and n.d. to 2.31 ng/L in July 2022.

3.2 Mortality of embryos and BSD score

Mortality and BSD scores did not differ significantly among the groups in either October 2021 or July 2022 (Figs. 2 & 3).

3.3 Biomarker response in embryos

Relative to the control, significant upregulation of the five gene transcripts was observed in embryos

Table 1 Concentrations of chemicals in surface seawater (ng/L)

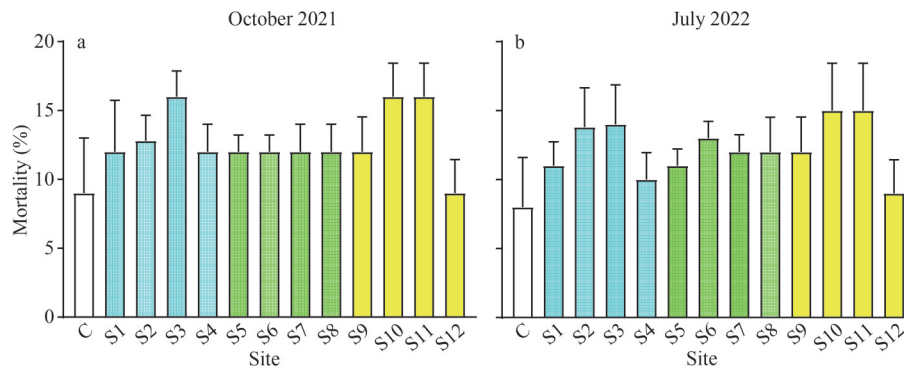
Site	October 2021			July 2022		
	4-NP	4- <i>t</i> -OP	BPA	4-NP	4- <i>t</i> -OP	BPA
C	n.d.	4.51	n.d.	n.d.	5.57	n.d.
S1	n.d.	25.16	8.99	6.93	10.77	n.d.
S2	n.d.	27.96	n.d.	7.83	5.60	n.d.
S3	n.d.	23.86	21.56	7.72	12.40	n.d.
S4	n.d.	24.44	n.d.	7.56	8.49	2.31
S5	n.d.	19.04	n.d.	7.47	7.30	n.d.
S6	n.d.	20.37	n.d.	8.35	8.09	1.14
S7	n.d.	22.17	n.d.	7.70	7.46	1.35
S8	n.d.	21.27	2.00	7.32	7.77	0.17
S9	n.d.	21.79	n.d.	7.29	5.94	2.31
S10	n.d.	26.56	n.d.	7.83	10.77	n.d.
S11	n.d.	23.12	n.d.	7.57	6.95	n.d.
S12	n.d.	24.26	n.d.	7.37	13.29	n.d.

n.d.: not detectable.

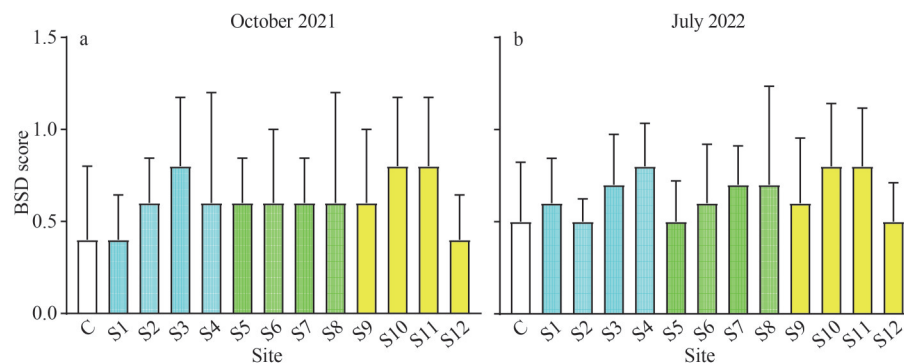
exposed to seawater from certain sampling sites in both October 2021 (Fig.4) and July 2022 (Fig.5). The highest transcript levels of *cyp19a* were found from S10 in October 2021 (Fig.4a) and S7 in July 2022 (Fig.5a), while the transcript levels of *cyp19b* were found from S11 in October 2021 (Fig.4b) and S10 in July 2022 (Fig.5b). The highest choriogenin (*chgL* and *chgH*) transcript levels were observed at S8 in October 2021 (Fig.4c) and S11 in July 2022 (Fig.5c), S11 in October 2021 (Fig.4d) and July 2022 (Fig.5d), respectively. A significant increase in *vtg* levels occurred in embryos from site S8 in October 2021 (Fig.4e) and site S10 in July 2022 (Fig.5e), relative to the control.

3.4 Value and graduated classification of IBR and IBRv2

In October 2021, the values of IBR and IBRv2 were higher in S8, followed by S11 (Fig.6a, b). In July 2022, the values of IBR and IBRv2 were higher

**Fig.2 Mortality of *O. melastigma* embryos following 96-h exposure to surface seawater from different sampling sites**

a. mortality in October 2021; b. mortality in July 2022. Columns: white: laboratory control; blue: sites in west of Kulangsu; green: sites in southeast of Kulangsu; yellow: sites in north of Kulangsu. Data are presented as mean±SE. No statistically significant differences were detected ($P \geq 0.05$).

**Fig.3 Embryonic BSD scores in *O. melastigma* following 96-h exposure to surface seawater from different sampling sites and their respective controls for 96 h**

a. BSD scores in October 2021; b. BSD scores in July 2022. Columns: white: laboratory control; blue: sites in west of Kulangsu; green: sites in southeast of Kulangsu; yellow: sites in north of Kulangsu. Data are presented as mean±SE. No statistically significant differences were detected ($P \geq 0.05$). BSD: blue sac disease.

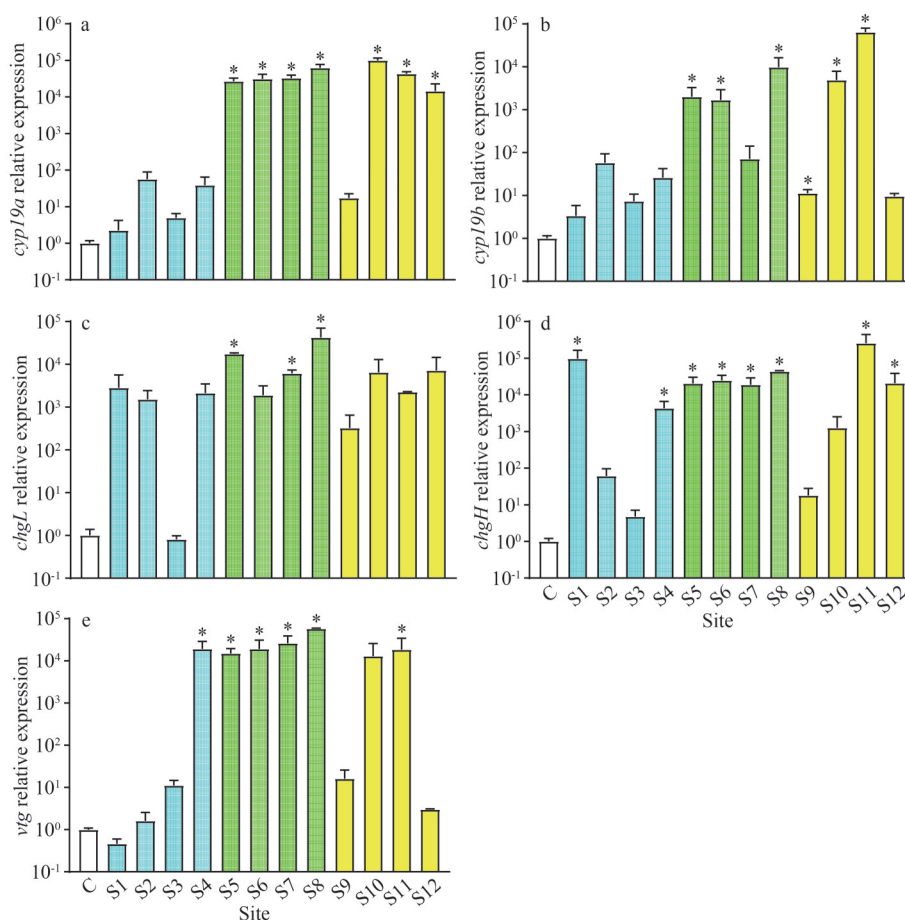


Fig.4 Relative mRNA expressions of five genes in *O. melastigma* embryos after a 96-h exposure to site-collected surface seawater (October 2021), compared to the control group

a. *cyp19a*; b. *cyp19b*; c. *chgL*; d. *chgH*; e. *vtg*. Columns: white: laboratory control; blue: sites in west of Kulangsu; green: sites in southeast of Kulangsu; yellow: sites in north of Kulangsu. Data are presented as mean $\pm$ SE. “*”: statistical significance was defined as $P < 0.05$.

in S11, followed by S10 (Fig.6c, d). The graduated thresholds of IBR and IBRv2 were calculated in accordance with the description provided in section 2.7.2. The graduated thresholds of IBR were 0.34 at Level II and 8.49 at Level III, while those of IBRv2 were 2.54 at Level II and 9.65 at Level III. In October 2021, the biological effect Level of S8 was ranked as Level III, whereas that of S5, S6, S7, S10, and S11 was classified as Level II based on the IBR algorithm. Application of the IBRv2 algorithm yielded a Level III classification for the biological effect levels at sites S5, S6, S7, S8, S10, and S11. In July 2022, the biological effect Level of S11 was determined to be Level III, whereas that of S10 was classified as Level II in accordance with the IBR algorithm. The biological effect levels of S10 and S11 were both classified as Level III according to IBRv2 algorithm.

4 DISCUSSION

4.1 Occurrence, distribution, and variation of chemicals

During October 2021 sampling, 4-NP was below detection limits at all sites, yet became detectable throughout the study area by July 2022. Within the Jiulong River estuary (JRE; sites S1–S4), mean 4-*t*-OP and BPA concentrations measured 25.36 and 7.64 ng/L (October 2021) and 9.32 and 0.59 ng/L (July 2022), consistently exceeded or were comparable to levels in nearby coastal seas (22.32-ng/L 4-*t*-OP, 0.26-ng/L BPA in October 2021; 8.45-ng/L 4-*t*-OP, 0.63-ng/L BPA in July 2022). This spatial gradient demonstrates riverine influence on contaminant distribution, with concentrations modulated by environmental variability and pronounced seasonal dynamics. Notably higher contaminant levels in the JRE versus coastal waters

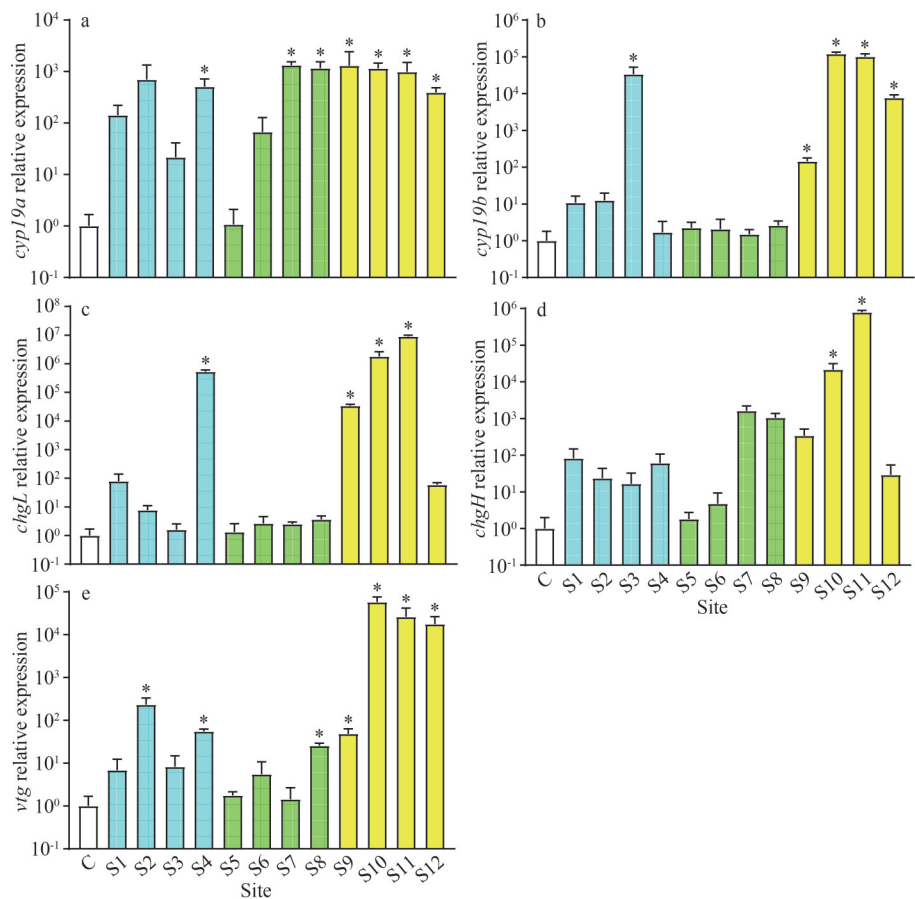


Fig.5 Relative mRNA expressions of five genes in *O. melastigma* embryos after a 96-h exposure to site-collected surface seawater (July 2022), compared to the control group

a. *cyp19a*; b. *cyp19b*; c. *chgL*; d. *chgH*; e. *vtg*. Columns: white: laboratory control; blue: sites in west of Kulangsu; green: sites in southeast of Kulangsu; yellow: sites in north of Kulangsu. Data are presented as mean $\pm$ SE. “*”: statistical significance was defined as $P < 0.05$.

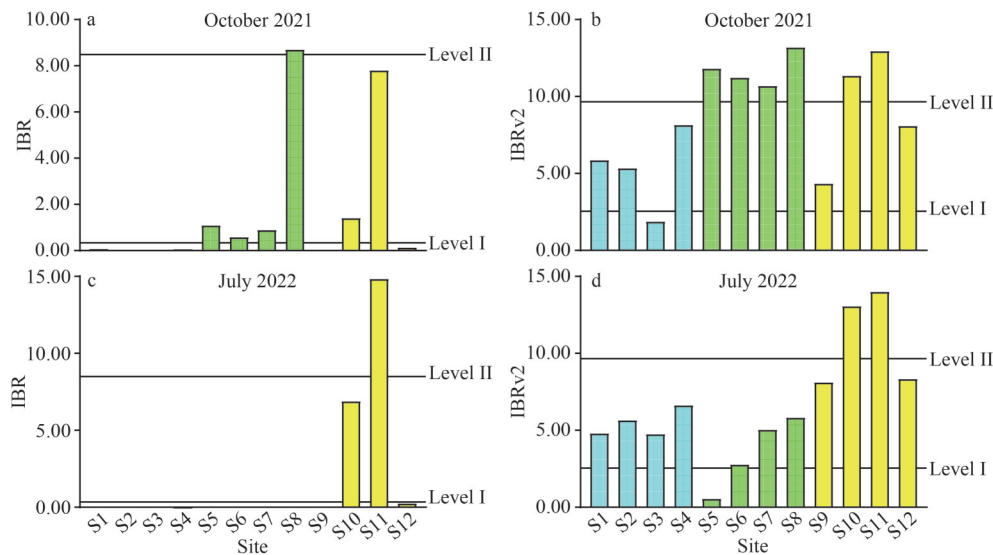


Fig.6 Quantitative comparison of IBR and IBRv2 metrics: value distributions and tiered classifications across biomonitoring sites

a. IBR in October 2021; b. IBRv2 in October 2021; c. IBR in July 2022; d. IBRv2 in July 2022. IBR: integrated biomarker response; IBRv2: integrated biomarker response version 2; Columns: white: laboratory control; blue: sites in west of Kulangsu; green: sites in southeast of Kulangsu; yellow: sites in north of Kulangsu.

confirm riverine runoff as a primary pollutant pathway to marine environments (Wu et al., 2019; Wang et al., 2022). The Xiamen Western Harbor (S10–S12) exhibited elevated 4-*t*-OP concentrations (24.65 ng/L in October 2021; 10.34 ng/L in July 2022) relative to nearby JRE coastal seas, attributable to concentrated anthropogenic sources including shipping operations and municipal-industrial effluent discharges, compounded by limited hydrodynamic flushing capacity (Wu et al., 2019; Zheng et al., 2024).

The global distribution of APs and BPA contamination in coastal waters has been systematically documented (Table 2). In this study, the concentrations of 4-NP in the JRE and nearby coastal seas ranged from n.d. to 8.35 ng/L, which were relatively lower than the levels recorded in notably polluted areas such as the coastal area of Ria de Aveiro, Portugal (Jonkers et al., 2010), Amur Bay, Russia (Cherniaev et al., 2016), Liaoning's coastal area, China (Liu et al., 2009), the Zhujiang (Pearl) River networks (Xie et al., 2020) and Thermaikos Gulf, Greece (Arditsoglou and Voutsas, 2012). In this study, the 4-NP concentrations were

also lower than those reported in other Chinese waters, including Taozi Bay, Sishili Bay, and Jiaozhou Bay (Huang et al., 2012). However, they were slightly higher than the concentrations in the Maowei Sea, China (Zheng et al., 2021).

For 4-*t*-OP, this study reported moderate levels (4.51–27.96 ng/L), which exceeded those in the coastal area of Ria de Aveiro, Portugal (Jonkers et al., 2010), Taozi Bay, Sishili Bay, and Jiaozhou Bay (Huang et al., 2012). These levels ranged from comparable to slightly exceeding those reported for the Maowei Sea, China (Zheng et al., 2021), the southern Bohai Sea, China (Deng et al., 2014) and Thermaikos Gulf, Greece (Arditsoglou and Voutsas, 2012). Comparatively, the Zhujiang River networks (Xie et al., 2020) displayed partial overlap with the range observed in this study.

BPA levels in this study (n.d.–21.56 ng/L) were lower than those in the southern Bohai Sea, China (Deng et al., 2014), Meizhou Bay, China (Hu, 2014), the Zhujiang River estuary, China (Zhao et al., 2019), the Maowei Sea, China (Zheng et al., 2021) and Tagus estuary, Portugal (Cunha et al., 2022). However, they were similar to the

Table 2 Regional variations in APs and BPA concentration in seawater samples around the world (unit: ng/L)

Location	4-NP	4- <i>t</i> -OP	BPA	Reference
JRE and nearby coastal seas, China	n.d.–8.35	4.51–27.96	n.d.–21.56	This study
Taozi Bay, China	n.d.–72.20	n.d.–2.10	n.d.–2.80	Huang et al., 2012
Sishili Bay, China	n.d.–75.60	n.d.–3.20	n.d.–36.40	Huang et al., 2012
Jiaozhou Bay, China	n.d.–212.90	n.d.–3.20	n.d.–36.60	Huang et al., 2012
Coastal area of Ria de Aveiro, Portugal	<29.00–78.00	<1.20–1.80	<1.10–170	Jonkers et al., 2010
Thermaikos Gulf, Greece	22.00–201.00	n.d.–18.20	10.60–52.30	Arditsoglou and Voutsas, 2012
Maowei Sea, China	n.d.	n.d.–6.64	6.57–90.82	Zheng et al., 2021
Amur Bay, Russia	n.d.–1240.00	–	–	Cherniaev et al., 2016
Coastal area of Liaoning, China	15.13–762.73	–	–	Liu et al., 2009
Zhujiang River networks, China	27.20–824.18	1.11–57.19	–	Xie et al., 2020
Meizhou Bay, China	n.d.–229.26	–	6.84–1 963.58	Hu, 2014
Southern Bohai Sea, China	–	<1.50–20.10	<1.50–275.00	Deng et al., 2014
Beibu Gulf, China	–	–	5.60–12.04	Gao et al., 2023
Port of Gdynia, Poland	–	–	n.d.–30.00	Bojke et al., 2023
Ross Island in the Antarctic	–	–	<1.30–29.5	Emnet et al., 2015
Laizhou Bay, China	–	–	7.60–152.00	Liu et al., 2013
JRE, China	–	–	11.10–101.00	Sun et al., 2016
Zhujiang River estuary, China	–	–	9.48–173.00	Zhao et al., 2019
Tagus estuary, Portugal	–	–	54.00–4 424.00	Cunha et al., 2022

n.d.: not detectable; –: not investigated.

concentration in the coastal area of Ria de Aveiro, Portugal (Jonkers et al., 2010), the port of Gdynia, Poland (Bojke et al., 2023), Ross Island (Emnet et al., 2015), and other Chinese waters such as Taozi Bay, Sishili Bay, and Jiaozhou Bay (Huang et al., 2012). Interestingly, the BPA concentrations in this study were much lower than those reported in a previous study conducted in the same region (Sun et al., 2016).

Generally, while these comprehensive comparisons highlight that the pollution levels of APs and BPA in the JRE and nearby coastal seas are relatively low to moderate compared to global hotspots, continued monitoring and management of these pollutants in the region are essential due to the significant impact of intense human activities.

4.2 Biomarker and integrated biomarker index

The absence of statistically significant changes in embryo mortality or teratogenic endpoints in this study is consistent with the moderate-to-low concentrations of APs and BPA detected by chemical analysis. Given these negative morphological outcomes, we subsequently examined molecular-Level responses through biomarker analysis to identify potential sublethal effects of APs and BPA exposure.

Numerous water samples collected in both October 2021 and July 2022 significantly induced the five selected estrogenic EDCs stress biomarkers (Figs.4 & 5). Previous studies have demonstrated that the mRNA expression of estrogen-responsive biomarkers, such as *cyp19*, *chgs*, and *vtg*, in fish embryos serves as a sensitive indicator of exposure to estrogenic EDCs (Schiller et al., 2014; Ishibashi et al., 2020). The biomarker response intensity follows a developmental gradient, with embryos showing greater sensitivity than larval and adult organisms (Peluso et al., 2021; Kishore et al., 2022). Similar, brain aromatase activities and plasma Vtg levels in male rockfish (*Sebastiscus marmoratus*) responded to estrogenic EDCs during a 2-week field monitoring study (Zheng et al., 2021). This study confirms that the expression of *cyp19*, *chgs*, and *vtg* in *O. melastigma* embryos serves as a highly sensitive biomarker for detecting estrogenic EDCs in the 96-h field monitoring study.

Exposure to seawater samples from sites S10 and S11 induced a significantly stronger response in these mRNA transcripts. The observed effects show clear correlation with the higher contaminant levels of 4-*t*-OP. However, although the concentrations of 4-*t*-OP and BPA in the JRE (sites S1, S2, S3, and

S4) were relatively high within the study region, no significant gene induction was observed—including *cyp19a*, *cyp19b*, and *chgL* in October 2021, as well as *chgH* in July 2022. The lack of observed effects may be due to some uncertain environmental factors, such as fluctuations in temperature, salinity, and pH in field study (Li et al., 2022; Slaby et al., 2024). Clearly, the salinity in the JRE was lower than in the nearby coastal seas, particularly in comparison to Xiamen Western Harbor (Supplementary Table S1).

Given that complex environmental conditions may confound biomarker interpretation, reliance on a single biomarker often proves insufficient for comprehensive environmental quality assessment. Consequently, a multi-biomarker approach provides a more robust assessment of environmental contamination (Garmendia et al., 2011). In this study, we implemented two integrated biomarker indices: the IBR and its updated version (IBRv2), which provide highly effective ways of approaching environmental toxicology (Nayak and Patnaik, 2021; Santos et al., 2021).

The most significant limitation of the IBR lies in the arbitrary sequencing of biomarkers within the radar diagram, which is typically user-defined without consistent theoretical justification. Therefore, the average IBR values were utilized to evaluate the overall biomarker response (Devin et al., 2014; Afssa et al., 2023; Passignat et al., 2025). Given the circular configuration of biomarkers in the radar diagram, the total number of distinct arrangements is reduced by half compared to linear permutations, as rotational duplicates are mathematically equivalent in this closed-loop system. Therefore, the number of distinct circular permutations for biomarker arrangements in the radar diagram equals $(n-1)!/2$, where n represents the number of biomarkers. The analysis resulted in a data matrix of 12 values for the five biomarkers (Table 3), half the size of the 24-permutation matrix reported by Devin et al. (2014).

While the IBR effectively quantifies mathematical relationships among biological data, it often fails to adequately interpret their underlying biological meaning (Ji et al., 2019). To prevent potential misinterpretations, environmental monitoring programs should incorporate biomarker responses in conjunction with chemical analytical data (Cravo et al., 2012). Consistent with the original IBR, IBRv2 demonstrates similar interpretative challenges. In this study, graduated classifications using both IBR

Table 3 The permutation patterns of five biomarkers in the radar diagram

Permutation pattern	Axis				
	1	2	3	4	5
1	A	B	C	D	E
2	A	B	C	E	D
3	A	B	D	C	E
4	A	B	D	E	C
5	A	B	E	C	D
6	A	B	E	D	C
7	A	C	B	D	E
8	A	C	B	E	D
9	A	C	D	B	E
10	A	C	E	B	D
11	A	D	B	C	E
12	A	D	C	B	E

The letters (A–E) denote the five biomarkers, respectively.

and IBRv2 revealed a greater number of sites categorized as Level II and Level III in October 2021 relative to July 2022 (Fig.6). These findings align with chemical analysis results demonstrating generally higher contaminant concentrations during the October 2021 sampling cruise compared to July 2022. Additionally, S10 and S11, which are located in narrow channels within the more polluted Xiamen Western Harbor, showed the highest frequency of Level II/III classifications in both the IBR and IBRv2 tiered-assessment approaches.

4.3 Tiered assessment in biomonitoring study

To systematically evaluate the environmental implications of IBRv2 values, a quantitative analytical framework was developed in our previous studies (Zheng et al., 2024). In this study, no Level IV or higher ratings were recorded. A Level III ranking (medium potential ecological risk) was identified at sites S5–S8, S10, and S11 in October 2021 and at S10 and S11 in July 2022, and this may be attributable to frequent anthropogenic activities, particularly shipping operations and tourism activities, in these locations (Wu et al., 2022).

Statistical analysis revealed consistent positive correlations between IBR and IBRv2 values across both sampling cruises (October 2021: $\rho=0.965$, $P<0.01$; July 2022: $\rho=0.895$, $P<0.01$). The results indicate concordant biological effect ordering between IBR and IBRv2 approaches. However,

attaining IBRv2 Level III classification requires the IBR framework to integrate all assessment results exceeding Level II thresholds. The IBRv2 index is calculated relative to a laboratory control group (X_0). Its thresholds (e.g., for ‘low’ or ‘moderate’ alteration) are therefore anchored to a specific, defined ‘unimpacted’ baseline. This makes IBRv2 thresholds biologically intuitive and absolute in the context of the experiment; a Level III classification signifies a deviation from this controlled reference condition that warrants management attention. In contrast, the IBR index is calculated based on the general mean (m_i) and standard deviation (s_i) across all field samples in a given dataset. Its thresholds are consequently relative and dataset-dependent. They define stress levels based on the distribution of responses within the monitored population of sites. An IBR Level II classification indicates a site’s stress level is statistically distinguishable from the central tendency of the surveyed area, serving as a precautionary, context-specific alert. Therefore, while both indices can achieve similar ordinal rankings, their thresholds answer different questions: IBRv2 thresholds answer “How impacted is this site compared to a clean reference?”, whereas IBR thresholds answer “How anomalous is this site’s stress level within the context of this specific monitoring campaign?”. This distinction is not a weakness but defines their respective applications. IBRv2 is powerful when a valid control exists, providing an absolute benchmark. IBR becomes essential when no pristine reference is available, offering a relative risk prioritization tool. Our results show that in a moderately impacted system, these different approaches can lead to convergent risk ordering but with different absolute threshold values.

This study demonstrates that both tiered biological effect assessment approaches maintain comparable validity amid environmental variability and seasonal pollutant fluctuations. Beyond methodological validation, the findings carry significant implications for environmental management. The tiered classification provides a direct link between biomarker responses and actionable risk levels. While both IBR and IBRv2 demonstrated high concordance in ranking sites, their differing classification outcomes suggest distinct practical warning thresholds, as evidenced by the more conservative Level III assignments typically made by IBRv2. For management purposes in environments like the JRE where establishing a reliable reference site is challenging, the IBR-based

approach requires that a Level II classification be treated as a significant precautionary alert, prompting increased monitoring vigilance and preliminary source investigation. In contrast, the control-based IBRv2 method designates Level III as a clear trigger for immediate attention and remedial action. This distinction is crucial for implementing adaptive management strategies that are tailored to the chosen biomonitoring tool.

Although this work represents a pilot study, it establishes a foundational framework for future research. Subsequent efforts should focus on generating larger datasets to: rigorously validate the reliability and consistency of both the IBR and IBRv2 tiered-assessment approaches, and to clarify their relationships with established chemical and ecological indices.

5 CONCLUSION

To our knowledge, this study is the first to apply IBR and IBRv2 tiered-assessment approaches to evaluate pollutant-induced biological effects in *O. melastigma* embryos in marine environments where environmental variability or seasonal contaminant fluctuations hinder reference site establishment. Our integrated assessment of the JRE and nearby coastal seas combined measurements of three EDCs, biomarker responses, and tiered IBR/IBRv2 evaluations. The results consistently identified sites S10 and S11 as the most heavily polluted areas, supported by elevated EDC concentrations and integrated biomarker responses. This study confirms that both IBR and IBRv2 are robust tools for marine biomonitoring and tiered biological effect assessment, with highly consistent conclusions. More importantly, it defines their respective roles in risk management: IBRv2 serves as a precise tool for scenarios with a valid control, where Level III dictates action, whereas IBR provides a vital early-warning framework for reference-site-limited environments, where Level II necessitates heightened vigilance. To address pollution at these priority sites, we recommend implementing source tracking, emission reduction strategies, and ecosystem restoration efforts. However, further validation is needed to assess the generalizability of these tiered-assessment approaches to other pollutants or ecosystems. Future research should focus on validating the tiered-assessment approaches with other pollutant classes (e.g., heavy metals, microplastics), species (e.g., bivalves, crustaceans), and ecosystems

(e.g., estuaries with different hydrological characteristics). Larger datasets will also be needed to clarify the relationships between IBR/IBRv2 and established chemical/ecological indices.

6 DATA AVAILABILITY STATEMENT

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

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Electronic supplementary material

Supplementary material (Supplementary Tables S1–S2) is available in the online version of this article at <https://doi.org/10.1007/s00343-026-5431-y>.