Acta Oceanol. Sin., 2024, Vol. 43, No. 6, P. 153–162
https://doi.org/10.1007/s13131-023-2245-z
http://www.aosocean.com
E-mail: ocean2@hyxb.org.cn

An innovative classification system for ranking the biological effects of marine aromatic hydrocarbons based on fish embryotoxicity

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Received 3 April 2023; accepted 8 September 2023

Abstract

Petroleum hydrocarbon pollution is a global concern, particularly in coastal environments. Polycyclic aromatic hydrocarbons (PAHs) are regarded as the most toxic components of petroleum hydrocarbons. In this study, the biomonitoring and ranking effects of petroleum hydrocarbons and PAHs on the marine fish model *Oryzias melastigma* embryos were determined in the Jiulong River Estuary (JRE) and its adjacent waters in China. The results showed that the levels of petroleum hydrocarbons from almost all sites met the primary standard for marine seawater quality, and the concentrations of the 16 priority PAHs in the surface seawater were lower compared with those in other coastal areas worldwide. A new fish expert system based on the embryotoxicity of *O. melastigma* (OME-FES) was developed and applied in the field to evaluate the biological effects of petroleum hydrocarbons and PAHs. The selected physiological index and molecular indicators in OME-FES were appropriate biomarkers for indicating the harmful effects of petroleum hydrocarbons and PAHs. The outcome of OME-FES revealed that the biological effect levels of the sampling sites ranged from level Ⅰ (no stress) to level Ⅲ (medium stress), which is further corroborated by the findings of nested analysis of variance (ANOVA) models. Our results suggest that the OME-FES is an effective tool for evaluating and ranking the biological effects of marine petroleum hydrocarbons and PAHs. This method may also be applied to evaluate other marine pollutants based on its framework.

Key words: petroleum hydrocarbons, polycyclic aromatic hydrocarbons, fish expert system, integrated biomarker response, nested one-way analysis of variance

Citation: Zheng Ronghui, Fang Chao, Hong Fukun, Zhang Min, Gao Fulong, Zhang Yusheng, Bo Jun. 2024. An innovative classification system for ranking the biological effects of marine aromatic hydrocarbons based on fish embryotoxicity. Acta Oceanologica Sinica, 43(6): 153–162, doi: 10.1007/s13131-023-2245-z

1 Introduction

With rapid industrialization development and energy demand around the world, petroleum hydrocarbon pollution in marine ecosystems is a major global concern due to its ubiquity, persistence, and potential toxicity to biota and human health (Bo et al., 2017; Yeo et al., 2017; Zhang and Yan, 2014). Estuaries receive pollutants as freshwater run-off from land use practices, and embayments are often the site of numerous harbors and industries. In particular, these waters are significantly affected by contamination with petroleum hydrocarbons. This is primarily due to the influx of oil from both land-based and marine sources such as municipal and industrial effluents, maritime ship traffic, oil drilling activities or accidental oil spills (Wang et al., 2021).

Petroleum hydrocarbons are composed of complex hydrocarbon mixtures, with varying solubilities depending on their octanol-water partition coefficients. Among them, polycyclic aromatic hydrocarbons (PAHs) are more soluble than alkanes with an equal number of carbon atoms. Consequently, some components of PAHs are regarded as the most toxic components of oil (Pasparakis et al., 2019; Zeng et al. 2015).

Biomonitoring of water quality is important in risk assessments of environmental pollution. Effect-based methods (EBMs), including cell-based bioassays, reporter gene assays and whole-organism assays, have been applied for decades in water quality biomonitoring (Escher et al., 2018) and have been applied in the early life stages of freshwater fish to differentiate good from poor water quality with respect to environmental chemical pollutants in surface waters (Cristiano et al., 2020; Tamura et al., 2017; Zhang et al., 2015) or in wastewater (Maier et al., 2015; Wittlerová et al., 2020).

In recent years, embryonic and larval developmental stages of fish are thought to be more sensitive to various environmental pollutants than later life history stages in EBMs studies (Fiorino et al., 2018; Mohammed, 2013; Pannetier et al., 2020). Many studies have shown that petroleum hydrocarbons and some PAHs can alter the normal early development of fish, mainly focus on

Foundation item: The Scientific Research Foundation of the Third Institute of Oceanography, Ministry of Natural Resources under contract Nos 2020014 and 2020017; the National Natural Science Foundation of China under contract No. 41977211; the National Program on Global Change and Air-Sea Interaction under contract No. GASI-02-SCS-YDsum.
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freshwater fish species such as common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) (Fiorino et al., 2018; Horie and Takahashi, 2021; Zhang and Yan, 2014), leading to a characteristic suite of pathologies, collectively referred to as blue sac disease (BSD). The syndromes of BSD include pericardial edema, yolk sac edema, cardiovascular disorders, craniofacial anomalies, spinal deformities, etc. (Carls et al., 2008; Gao et al., 2018; Mcintosh et al., 2010).

Marine medaka (*Oryzias melastigma*) have been recommended as an important marine fish model for ecotoxicology studies due to their small size, short generation cycle, high spawning rate, simple culture conditions and abundant genetic information (Bo et al., 2011; Dong et al., 2014; Kong et al., 2008). However, few studies have documented the application of EBMs in coastal water quality biomonitoring with *O. melastigma* larvae or marine fish embryos (Horie and Takahashi, 2021).

Furthermore, based on the simple analysis of the simultaneous changes in different physiological parameters, it is generally difficult to obtain a correct evaluation of the overall changes in the health status of organisms stressed by environmental pollutants. The recent development of potential algorithms can integrate information derived from the responses of multiple biomarkers and should allow an objective interpretation of ecotoxicological data (Wan et al., 2018). In our previous study, a new fish expert system (FES) with different levels of biological organization (i.e., molecular/cellular, tissue and organism) affected by pollutants was developed for evaluating the biological effects of PAHs on the rockfish *Sebastiscus marmoratus* (Zheng et al., 2022).

The Jiulong River Estuary (JRE) is a typical shallow macrotidal estuary with high turbidity in the low salinity region, connecting the Jiulong River watershed and the Taiwan Strait. It covers an area of 100 km^2 and is approximately 21 km long from east to west and 6.5 km wide from north to south (Wu et al., 2017). The annual average riverine runoff into the JRE is 1.48×10^{10} m^3, with 74% being discharged in the wet season (April to September) and 26% in the dry season (October to March) (Chen et al., 2014). Marine pollutants (including petroleum hydrocarbons and PAHs) from the Jiulong River and the south sea area of Xiamen is easy to affect the west sea area of Xiamen in flood tide (Chen et al., 2014; Gong, 2009; Wu et al., 2011, 2017). In addition, the JRE experiences frequent shipping activities and domestic and industrial sewage discharge from the west sea area of Xiamen due to the rapid economic development of Xiamen in past four decades (Yan et al., 2012; Wu et al., 2016, 2019).

In this study, we examined the impact of early-life exposure to seawater on *O. melastigma* embryos, investigating effects ranging from subcellular effects to whole-organism responses. Specifically, the objectives of this study were to (1) evaluate the levels of petroleum hydrocarbons and PAHs in the surfer seawater from the JRE and its adjacent waters in China; (2) analyze the effect of sampling area differentiation on the biological effects of petroleum hydrocarbons and PAHs; and (3) develop a new FES based on *O. melastigma* embryos (OME-FES) to rank the biological effects of petroleum hydrocarbons and PAHs in this coastal region.

2 Materials and methods

2.1 *Study area and sampling*

Different sampling sites were in the JRE and its adjacent waters (Fig. 1). The sites were divided into three areas based on different human activities: the estuary area (including four sites, E1–E4), the tourism area (including four sites, T1–T4) mainly with coastal tourism and domestic activities, and the port area (including four sites, P1–P4) mainly with maritime transportation activities.

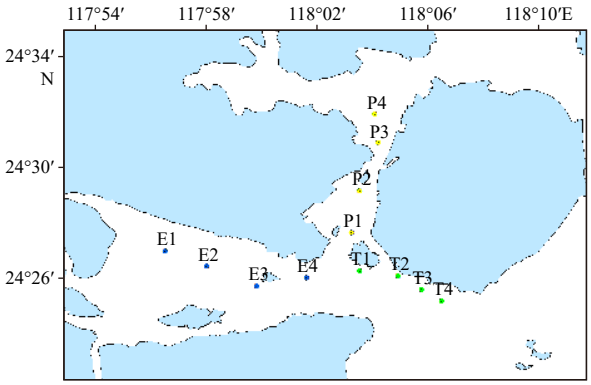

Fig. 1. Study area and sampling sites.

Surface seawater samples were collected directly from 0.5 m below the water surface using a Niskin rosette at the 12 sites in October 2021 (dry season). The samples were then stored at precleaned solvent-rinsed brown glass bottles and refrigerated at 4℃ until chemical extraction, which was conducted within a few hours. The surface seawater samples for embryo exposure were stored in 10 L bisphenol A-free, opaque, food grade plastic buckets.

2.2 *Chemical analysis*

The concentrations of petroleum hydrocarbons and 16 priority PAHs defined by the US Environmental Protection Agency (EPA) (Table S1) in the surface seawater samples were analyzed by a gas chromatograph-mass spectrometry (Agilent 7000C) with a fused silica column (HP-5 ms, 30 m × 0.25 mm i.d.× 0.25 μm film thickness, J & W Scientific) as described in a previous study (Bo et al., 2017). In addition, the concentrations of seven indicator PCB congeners (PCB28, PCB52, PCB101, PCB118, PCB138, PCB153, and PCB180) suggested by the International Committee for the Exploration of the Sea (ICES), hexachlorocyclohexanes (HCHs) (α-, β-, γ-, and δ-HCH), and dichlorodiphenyltrichloroethanes (DDTs) (p, p′-DDE, p, p′-DDD, o, p′-DDT, and p, p′-DDT) in the surface seawater were measured to better understand the potential impact on the fish described by Bo et al. (2017). Procedural blanks were set up to check for interference. The target compounds were undetectable in the blank during the whole experiment. Internal standards (pyrene-d10 and PCB 103) were employed to calculate surrogate recoveries, which ranged from 76.8% to 103.1% for PAHs and 75.6% to 109.3% for PCBs and organochlorine pesticides (OCPs).

2.3 *Fish maintenance*

The marine medaka *O. melastigma* were obtained from the City University of Hong Kong, and standard operating procedures for largescale culturing of *O. melastigma* were performed as previously established (Bo et al., 2011). The preparation of fish embryos was conducted following a previous study (Zhang et al., 2021). In brief, adult *O. melastigma* were transferred into a breeding aquarium at a ratio of 1:1 male:female. Breeding crosses were set at 17:30, and eggs were collected the following morning between 8:00 and 9:00. The collected eggs were observed under a stereomicroscope (Nikon SMZ1270; Nikon Corp., Tokyo, Japan) to distinguish the unfertilized eggs, ensuring that the eggs used in the experiment developed well. The collected fertilized eggs were

then randomly distributed into glass petri dishes with a 20 cm diameter and incubated in aerated artificial seawater with a salinity of 30 at 28℃. After 48 h of cultivation, healthy embryos were selected for embryo exposure. All animal experiments were conducted in compliance with the guidelines set by the Xiamen University Institutional Committee for the Care and Use of Laboratory Animals (XMULAC20190066).

2.4 *Embryo exposure and sampling*

Embryo exposure experiments were conducted for 4 d between 3 d and 6 d post fertilization (dpf). This early-life exposure window included two important early-life stages of *O. melastigma* (Chen et al., 2020): (1) 3 dpf (from early morula stage to late blastula stage; the heart is well developed) (Mu et al., 2011) and (2) 6 dpf (liver is well developed).

Then, 100 mL surface seawater samples at different sites were added to glass petri dishes with a 12 cm diameter. Artificial seawater was used as a blank control (C). One hundred healthy embryos were randomly selected and transferred into glass petri dishes with the above solutions. All treatments were performed in five biological replicates. Two-thirds of the test solution in each dish was replaced daily with the same seawater samples during the experimental period.

The pooled samples of 15 embryos after 4 d of exposure from each duplicate were frozen immediately in liquid nitrogen and stored at –80℃ to determine the mRNA expression levels of target genes (refer to Section 2.6). During the exposure period, dead embryos were immediately removed from the petri dishes and recorded. Meanwhile, the heart rate and malformation data were recorded daily.

2.5 *Teratogenicity assessment*

The teratogenicity of the surface seawater samples to the embryo was assessed using the BSD score. The calculation of BSD was the method described by Gao et al. (2018) and Wu et al. (2012) with minor modifications. In each embryo, signs were scored according to the following parameters: “0” if there were no presence/absence signs; “1” if there were presence/absence signs, e.g., abnormal heart rate, heart malformations, loss of activity, spinal deformity, craniofacial deformity, fin rot, and yolk/body hemorrhaging; and “3” if there were severe signs, e.g., yolk sac edema and pericardial sac edema. The scores for yolk sac edema and pericardial sac edema were higher compared to other signs, as edema was most prominently associated with mortality. The BSD score was calculated as the average of the summed scores for each fish across all signs of toxicity within each group.

The abnormal heart rate was measured by recording the time required to reach 30 beats in the treatment groups compared to the control group. The time to reach 30 beats for each fish embryo was converted to the heart rate in beats per minute using the following formula: $N = 30/T \times 60$, where N represents the number of heart beats per minute of embryo (unit of beats/min) and T represents the time required to reach 30 embryo beats.

2.6 *Real-time quantitative PCR (RT-qPCR) analysis of the target genes*

The expression levels of the following genes were measured by RT-qPCR: (1) three cytochrome P450 superfamily (CYP) genes that are associated with phase I xenobiotic and drug metabolism (CYP1A1, CYP27B and CYP3A40) (Chen et al., 2020; Zhang et al., 2017) and (2) two transcription factors involved in the regulation of detoxification genes (AhR and ARNT) (Chen et al., 2022; Zhou et al., 2020). 18S ribosomal RNA (18S rRNA) was used as the housekeeping gene. The primer sequences utilized in this study are listed in Table S2. The amplification efficiency of all the tested genes ranged from 90% to 120%. RT-qPCR was performed using a Rotor-GeneQ fluorescent quantitative thermal cycler (Qiagen, Germany), and the data analysis was carried out as previously described (Bo et al., 2012).

2.7 *Development of a fish expert system based on O. melastigma embryos*

The OME-FES was developed to evaluate the biological effects of petroleum hydrocarbons and PAHs on *O. melastigma* embryos. The frame of the OME-FES was designed based on our previous reports (Zheng et al., 2021, 2022) with modifications as follows. The final estimation of the biological effect level on *O. melastigma* embryos was mainly based on (1) the different biological levels affected by petroleum hydrocarbons and PAHs (i.e., molecular/cellular, tissue and organism); (2) the response patterns (i.e., increasing, decreasing, bell-shaped) of each selected biomarker; and (3) the magnitude of the alteration of the integrated biomarker response version 2 (IBRv2) (Sanchez et al., 2013) values based on the selected biomarkers.

2.7.1 *Determination of the biological effect index*

OME-FES determines a biological effect index (BEI), ranking biological effects according to a 5-tier scale, from level Ⅰ (no stress) to level Ⅴ (survival stress). The stress level is determined by considering various biological levels affected by petroleum hydrocarbons and PAHs and comparing the IBRv2 values (refer to Section 2.7.2) with the modified alteration levels (refer to Section 2.7.3): first, if the treatment group shows statistically significant differences at the organism level (significantly decreased survival rate) compared with the control, then its BEI was ranked as level Ⅴ (survival stress); second, if the treatment group does not show alterations at the organism level but at the tissue level (significantly increased BSD score), then its BEI was ranked as level Ⅳ (high stress); third, if the treatment group does not show alterations at the tissue/organism level, but only at the molecular/cellular level, then its BEI was ranked as level Ⅰ (no stress), level Ⅱ (low stress) or level Ⅲ (medium stress) by comparing the IBRv2 values (see Section 2.7.2) with the modified alteration levels (see Section 2.7.3).

2.7.2 *Calculating the IBRv2 values of the sensitive biomarkers*

The IBRv2 calculation followed the description in our previous study (Zheng et al., 2022).

2.7.3 *Determination of the alteration levels of the sensitive biomarkers*

The biomarker data were converted into one of three alteration levels (AL) (from NA, i.e., no alteration, to ++/––, i.e., moderate alteration), following modification and refinement of the alteration levels and biological relevance that had been developed previously for marine mussels (Dagnino et al., 2007) (refer to Table 1). Critical values (CV), representing varying degrees of severity from normal reference responses, were calculated following the methodologies described by Wan et al. (2018) and Zheng et al. (2022):

$$\mathrm{CV_n} = m_\mathrm{c} \times \beta_\mathrm{n}, \tag{1}$$

where m_c is the data of the selected biomarkers in the control. β_n is the threshold value of the alteration level for the selected bio-

Table 1. Determination of the alteration level (AL)

Decreasing parameter		Increasing and bell-shaped parameter		Biological relevance
Threshold	AL	Threshold	AL	
AF > 0.8	NA	AF < 1.2	NA	Small differences (±20%) with respect to controls; although statistically significant, they are not considered of biological relevance.
AF < 0.8	–	AF > 1.2	+	Larger than 20%, statistically significant differences with respect to controls. The symbols "–" and "+" indicate decreased or increased physiological responses of the organisms, respectively.
AF < 0.5	– –	AF > 2.0	++	Large differences (>100%) with respect to controls. The symbols "––" and "++" indicate moderate decreased or increased physiological responses of the organisms, respectively, which are within the range of alterations induced by moderate natural stressors.

Note: AF, alteration factor; NA, no alteration.

marker response, which is selected according to the alteration factor (AF) in Table 1. If the selected biomarker shows a decreasing response, then β_n is 0.80 and 0.50 in sequence; otherwise, β_n is 1.20 and 2.00. Thus, calculating the alteration level based on the CV_n of the sensitive biomarkers is the same as that of IBRv2. The modified alteration level was then divided into three categories that were the IBRv2 of each AL.

2.8 *Statistical analyses*

All results are reported as the mean ± standard error (S.E.). The statistical analysis was performed with SPSS v26.0 (SPSS Software Inc., USA). The normal distribution of the data was initially assessed using Shapiro-Wilk's test, followed by Levene's test to evaluate the homogeneity of variances. Then, to identify significant differences in the data between the treatment groups and the control group, one-way analysis of variance (ANOVA) was assessed, and a post hoc comparison was applied to test the significance of each dataset (Student-Newman-Keuls's test for homogenous data or Dunnett's T3 test for nonhomogenous data). In the case where datasets did not meet assumptions of normality, the Kruskal-Wallis ANOVA was used. Spearman's correlation analysis was applied to compare relationships between IBRv2 values and chemical variables. Nested ANOVAs were performed for IBRv2 values to test the null hypothesis that means do not differ among the three areas. Significant differences were accepted at * $p < 0.05$. Graphs were generated using GraphPad Prism v8.4.

3 Results

3.1 *Concentrations of PAHs and petroleum hydrocarbons in seawater*

Concentrations of Σ_{16} PAHs in the water phase ranged from 20.22 ng/L (C) to 38.71 ng/L (P4) with an average of (31.00 ± 5.58) ng/L (Table S1). The individual PAH congener with the highest concentration in the surface seawater was naphthalene (Nap) at all sampling sites. The following nine individual congeners were detectable at all sites: naphthalene (Nap), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), fluoranthene (Fla), and pyrene (Pyr). Acenaphthylene (Acy) was detectable at all sites except E3, T2, T3 and T4. The distribution of the PAHs in the seawater based on the different ring numbers is illustrated in Fig. 2. Low molecular weight (LMW) PAHs (2- and 3-ring) were dominant in the seawater from each sampling site, accounting for 79%–90% of the total PAHs.

The concentrations of petroleum hydrocarbons in the seawater ranged from 3.1 μg/L (C) to 54.9 μg/L (P3), with an average of (23.44 ± 12.05) ng/L (Table S1). In addition, the HCHs (sum of α-, β-, γ-, and δ-HCH), DDTs (sum of 2, 4′-DDT, 4, 4′-DDT, 4, 4′-DDD, and 4, 4′-DDE) and 2 indicator PCB congeners (PCB138 and PCB153) were nondetectable in the present study, and the concentrations of PCBs ranged from nd (C) to 2.75 ng/L (P2).

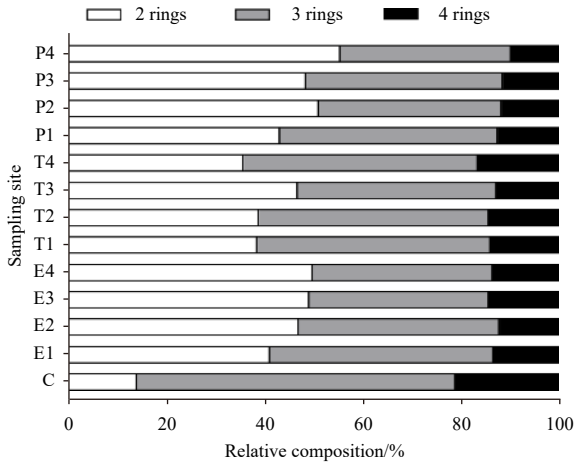

Fig. 2. Composition of PAHs according to different ring numbers in seawater from each site.

3.2 *BSD score and mortality of embryos*

There were no significant differences in mortality or BSD scores between the treatment groups and the control group (Figs S1 and S2).

3.3 *Biomarker responses in embryos*

Significant increases in CYP1A1, CYP27B, CYP3A40, AhR and ARNT mRNA expression levels were observed at various sites in the three study areas compared with the control group, especially in the tourism area and the port area (Fig. 3). The highest mRNA expression levels of all the tested genes were found at P3 (Fig. 3). The star plots showed the deviations in the IBRv2 values compared with the embryos from the control group (Fig. 4), and the IBRv2 values of the embryos from the sites in decreasing order were determined as follows: P3 (16.63) > P2 (12.34) > T1 (10.72) > T4 (9.57) > T3 (9.14) > E4 (7.42) > T2 (5.38) > E2 (5.36) > P4 (5.18) > E3 (3.52) > P1 (3.36) > E1 (1.69). Nested ANOVA models compared to IBRv2 indicated significant differences in the study areas, and there were also significant differences between each pairwise comparison (Table 2).

3.4 *Assessment of biological effects*

To assess the biological effects of petroleum hydrocarbons and PAHs in the surfer seawater from the JRE and its adjacent waters on *O. melastigma* embryos, the data were analyzed as described in Section 2.7. First, the response profile of the biomarkers and the level of biological organization were determined according to previous studies (Chen et al., 2020, 2022; Zhang et al., 2017; Zhou et al., 2020) (Table 3). Second, alterations at the tissue/organism level were not found in embryos from any sites; alterations were found only at the molecular/cellular level. Therefore, the BEI ranges from level Ⅰ (no stress) to level Ⅲ (medium

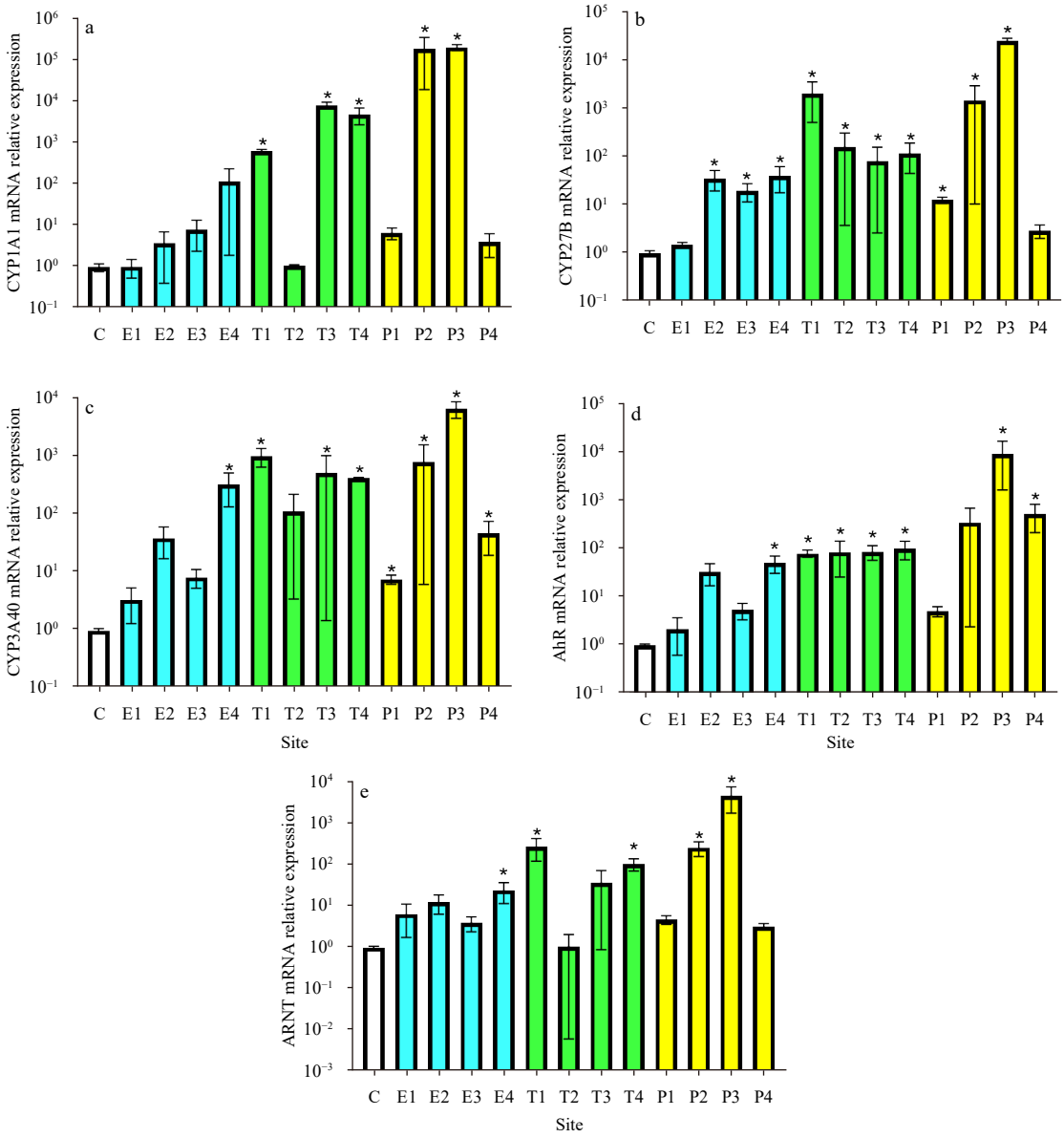

Fig. 3. Relative mRNA expression levels of five target genes in the embryos treated with surface seawater samples at different sites compared with the control after 4 d exposure. Data are expressed as the mean ± standard error. Significant differences were accepted at * $p < 0.05$. Five biological replicates were conducted.

stress). Third, the alteration levels based on the CV of the sensitive molecular/cellular biomarkers were calculated to be the same as those in IBRv2 (Table 4). Finally, by comparing the IBRv2 index of each site (Fig. 4) with the threshold values of the BEI (Table 4), the BEI of site E1 was ranked as level Ⅰ (no stress), the BEIs of sites E2, E3, E4, T2, T3, T4, P1, and P4 were all ranked as level Ⅱ (low stress), and the BEIs of sites T1, P2, and P3 were ranked as level Ⅲ (medium stress).

4 Discussion

4.1 *Occurrence and sources of petroleum hydrocarbons and PAHs*

In this study, the levels of petroleum hydrocarbons from all sites, except for P3, met the primary standard for marine seawater quality GB 3097–1997 (National Environmental Protection Administration, 2004), which was issued to protect habitats for marine life, including natural, rare and endangered species, as well as places for recreation. However, the level of petroleum hydrocarbons at site P3 exceeded the first class of criterion, thereby indicating potentially negative effects. This finding is consistent with reports on the average concentrations of petroleum hydrocarbons in the JRE and its adjacent waters (Guo et al., 2012; Wei, 2014). The petroleum hydrocarbons could have been derived from riverine input, frequent shipping and emissions of industrial wastewater and domestic sewage in the study areas (Guo et al., 2012; Wu et al., 2019).

The concentrations of Σ_{16} PAHs in surface seawater were lower compared with the data from other coastal waters worldwide (Table 5). The concentrations of DDTs and HCHs were lower than the detection limits, and the levels of PCBs were very low or nondetectable in the sampling sites, which suggested that petroleum hydrocarbons and PAHs in seawater may largely contribute to the induced biological effects on *O. melastigma* embryos in this study.

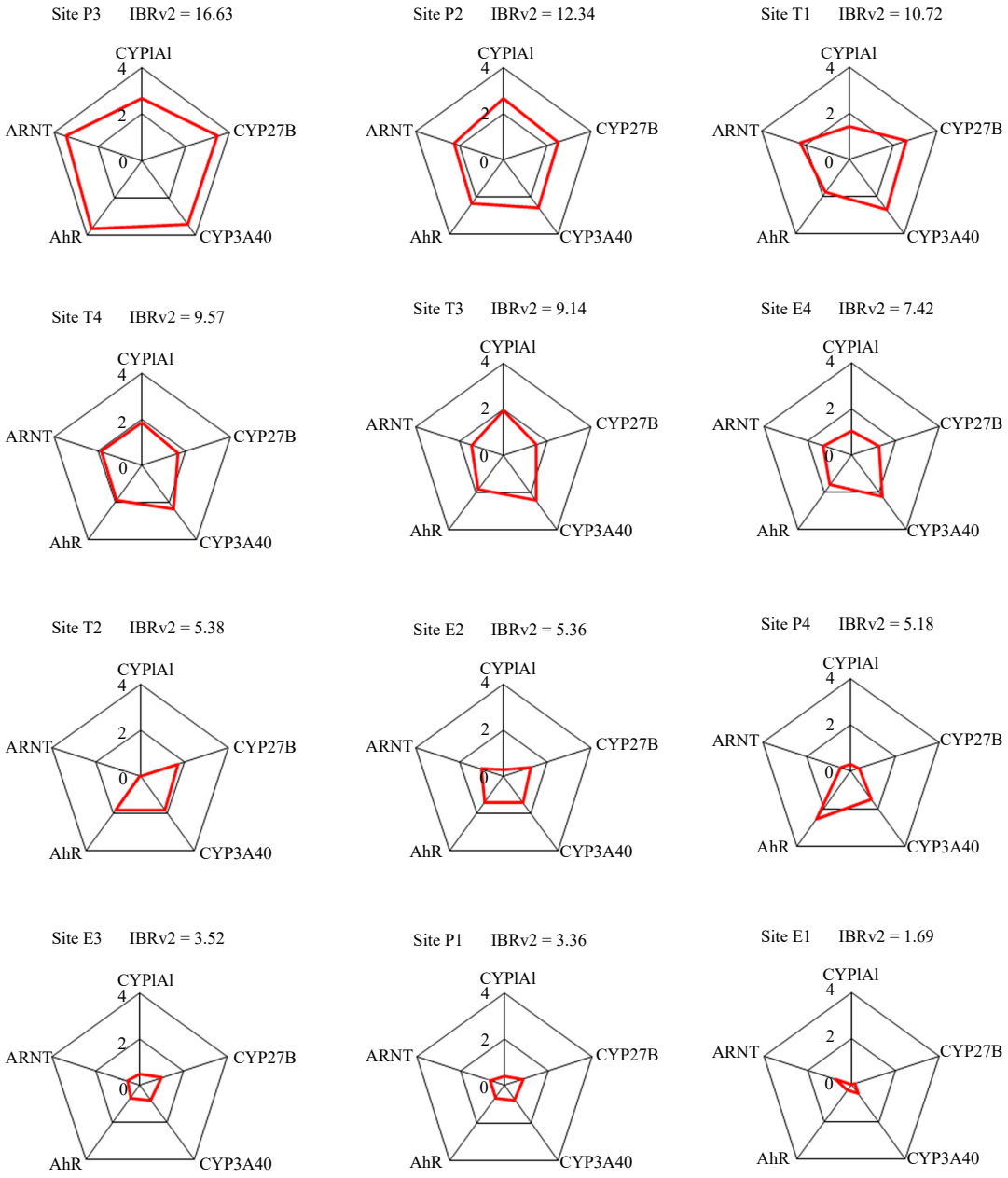

Fig. 4. Star plot and IBRv2 values at each sampling site. The order of each site was arranged according to the IBRv2 values, which was decreased from left-to-right and top-to-bottom.

Table 2. Summary results of nested ANOVA analyses for IBRv2 values

Source	DF	SS	MS	*F*	*p*
Study areas	2	135.869	67.935	1 811.151	0.000
E-T	1	93.268	93.268	4 198.732	0.000
E-P	1	112.483	112.483	2 134.084	0.000
T-P	1	2.273	2.273	57.537	0.000

Note: E, the estuary area; T, the tourism area; P, the port area; DF, degrees of freedom for each component; SS, the sum-of-squares; MS, the mean-squares; *F* and *p* for the hypothesis test of significant study area heterogeneity.

Table 3. Response profiles of the biomarkers selected in OME-FES and their levels of biological organization

Biomarker	Response profile	Level of biological organization
CYP1A1 gene	increasing	molecular/cellular
CYP27B gene	increasing	molecular/cellular
CYP3A40 gene	increasing	molecular/cellular
AhR gene	increasing	molecular/cellular
ARNT gene	increasing	molecular/cellular
BSD score	increasing	tissue
Survival rate	decreasing	organism

4.2 *Implications of the changes in biomarkers and IBRv2 values in field monitoring*

The values of IBRv2 and the BEI are calculated based on biomarkers. Therefore, it is critical to select sensitive biomarkers for OME-FES. In this study, a battery of sensitive biomarkers was employed to evaluate the biological effects of petroleum hydro-

Table 4. Critical values of five selected biomarkers and the threshold values of BEI

	Control	β = 1.20	β = 2.00
CYP1A1 gene	1.00	1.20	2.00
CYP27B gene	1.00	1.20	2.00
CYP3A40 gene	1.00	1.20	2.00
AhR gene	1.00	1.20	2.00
ARNT gene	1.00	1.20	2.00
Threshold values of BEI	0.00	2.54	9.65

Note: β: biomarkers and threshold values selected according to the alteration factor in Table 1 and response profiles of the molecular biomarkers in Table 3.

carbons and PAHs on *O. melastigma* embryos.

Similar to the results obtained in the present study, variations in the mRNA expression patterns of the CYP1A1, CYP27B, CYP3A40, AhR and ARNT genes were also observed in many fishes, such as flounder (*Platichthys flesus*), rockfish (*S. marmoratus*), and marine medaka (*O. melastigma*), both in laboratory and field studies (Bo et al., 2017; Chen et al., 2020, 2022; Reynaud et al., 2008; Reynolds et al., 2003; Zhang et al., 2017; Zhou et al., 2020). These findings strongly suggest that these genes play critical roles in xenobiotic detoxification in marine fishes, including *O. melastigma*, which can serve as sensitive biomarkers for the risk assessment of organic xenobiotics in aquatic environments.

In a technical report of the Water Framework Directive (WFD-2000/60/EC), the adoption of apical short-term ecotoxicity bioassays, i.e., fish embryo toxicity and immobilization of *Daphnia* sp., is recommended as aquatic EBMs (Brack et al., 2019; Cristiano et al., 2020; Wernersson et al., 2015). The study reported by Horie and Takahashi (2021) was the first one to use *in vivo* acute bioassays with *O. melastigma* larvae as an EBM to monitor the toxicity of triclosan, 3, 4-dichloroaniline, and pyriproxyfen in seawater. The result showed that the toxicity of triclosan and pyriproxyfen differed between marine medaka (*O. melastigma*) and freshwater medaka (*O. latipes*) underlines the importance of using marine organisms to evaluate the ecological effects of chemicals in marine environment (Horie and Takahashi, 2021). The present results suggest that *O. melastigma* embryo is a potential EBM to monitor seawater quality.

The IBRv2 values can provide insights into the actual adverse impacts of contamination by petroleum hydrocarbons and PAHs on *O. melastigma* embryos. Indeed, the IBRv2 values were positively correlated with the concentration of petroleum hydrocarbons in the surface seawater (ρ = 0.832, p < 0.05). The responses of biomarkers and IBRv2 values suggested that the embryos from site P3 experienced the most deleterious effects associated with contamination by petroleum hydrocarbons and PAHs. Furthermore, nested ANOVAs suggested that the study area affected the toxicity of petroleum hydrocarbons and PAHs by comparing IBRv2 values among the investigated areas. Thus, diverse human activities in different study areas can cause various toxic effects of petroleum hydrocarbons and PAHs. The pollution of petroleum hydrocarbons and PAHs could have been generated by diverse sources in the present study (Chau, 2006; Li et al., 2014; Wu et al., 2019), and thus, source control enforcement and energy structure optimization should be considered in these areas as well as other coastal regions (Fang et al., 2020; Sun et al., 2016; Yu et al., 2016).

4.3 *Environmental implication of the results of the OME-FES*

IBRv2 values represent a sum of deviations between reference and measured values. The higher the IBRv2 value, the greater the risk of contaminant on the tested organisms. To distinguish the environmental implication of the IBRv2 values, quantitative methods are urgently required (Wan et al., 2018; Zheng et al., 2021, 2022). In addition, it is also necessary to link the observed effects with the cost-effective management objectives. Therefore, to further study the significant differences among the three study areas, OME-FES was developed to rank the biological effects of petroleum hydrocarbons and PAHs on *O. melastigma* embryos. The results demonstrated that the environmental stress of two sites in the port area was ranked as level Ⅲ (medium stress), while that of one site in the tourism area and that of no site in the estuary area were ranked as level Ⅲ. Moreover, the only site in which BEI ranked as level Ⅰ (no stress) was in the es-

Table 5. Concentrations (ng/L) of ΣPAHs in coastal waters from different areas worldwide

Sampling area	Number of PAHs	*n*	Mean	Min	Max	Reference
JRE and its adjacent waters	16	12	31.0	20.22	38.71	this study
Langkawi Island, Malaysia	18	–	–	18.0	46.0	Zong et al., 2014
Natuna, Indonesia	17	–	–	0.0	5.8	Zong et al., 2014
Oostende, Belgium	15	–	–	13.0	24.0	Zong et al., 2014
Chesapeake Bay, USA	17	38	33.3	20.0	65.6	Zhou and Maskaoui, 2003
Dalian coastal area, China	9	17	60.8	50.5	74.7	Zhang et al., 2020
Maowei Sea, China	16	10	–	33.5	48.5	Zheng et al., 2022
Western Taiwan Strait, China	15	31	19.8	12.3	58.0	Wu et al., 2011
Xiamen Bay, China	16	17	17.0	7.0	26.9	Maskaoui et al., 2002
JRE, China-wet season	46	20	67.1	24.6	125.9	Wu et al., 2019
JRE, China-dry season	46	20	27.4	17.5	65.0	Wu et al., 2019
Thane creek, India	16	10	–	337	706	Tiwari et al., 2017
Persian Gulf, Iran	30	360	464	70	884	Jafarabadi et al., 2017
Aegina Island, Greece	17	–	–	103	124	Zong et al., 2014
Dalian coast, China-winter	46	15	357	136	621	Hong et al., 2016
Dalian coast, China-summer	46	15	297	65	1 130	Hong et al., 2016
Luan River Estuary, China	14	9	–	231	3 664	Yan et al., 2016
Hai River Estuary, China	14	11	–	288	3 797	Yan et al., 2016
Zhangweixin River Estuary, China	15	10	–	306	7 597	Yan et al., 2016

Note: JRE: Jiulong River Estuary; *n* denominates the number of samples; – represents no data.

tuary area. Thus, the results of nested ANOVA models were verified by OME-FES. In particular, sites P2 and P3, which were categorized by BEI as level Ⅲ, were situated in narrow waters in the west sea area of Xiamen with heavy maritime transportation. The possible reason for the most harmful effects at these two sites was the poor self-purification capacity in the west sea area of Xiamen, especially when pollution occurs at low tide (Gong, 2009). Very close to the effluent discharge outlet of a sewage treatment plant on Kulangsu Island, which might be the reason why the BEI of site T1 was categorized as level Ⅲ. The differences in IBRv2 values and BEI between the upstream and downstream JRE may be due to different hydrodynamic exchange conditions or the new input sources emitted from Xiamen Harbor to the JRE in the dry season (Wu et al., 2019).

In addition, the BSD score and five related gene expression levels were utlized in the classification of level Ⅳ and level Ⅰ to level Ⅲ instead of the hepatosomatic index (HSI), gonadosomatic index (GSI), hepatic ethoxyresorufin-*O*-deethylase (EROD) activities, and biliary fluorescent aromatic compounds (FACs) in adult rockfish *S. marmoratus* in Zheng et al. (2022). The application of these indices in this study not only better suits the characteristics of embryos but also enables the grading indicators of level Ⅳ more accurate. Our findings show that OME-FES is an effective tool in environmental risk assessment studies of petroleum hydrocarbons and PAHs. The features of OME-FES, especially its extendibility and its capacity for summarizing information derived from different biological data in aromatic hydrocarbon pollution, make this system useful not only for environmental scientists but also for environmental policymakers. Furthermore, new classification systems for ranking the biological effects of other pollutants are worth expecting by following the framework of this classification system through selecting specific and sensitive biomarkers for specific pollutants.

5 Conclusions

In this study, we determined the contamination levels, sources of petroleum hydrocarbons and PAHs, and hazard levels in *O. melastigma* embryos in the JRE and its adjacent waters by combining chemical monitoring with the OME-FES approach. Our results indicated that the concentrations of the 16 priority PAHs in the surface seawaters were lower compared with those in other coastal areas worldwide. There are significant differences in the IBRv2 values among the three study areas by nested ANOVA. The result of nested ANOVAs was further confirmed by the outcome of OME-FES. Generally, the sampling sites experienced no stress to medium stress according to OME-FES. The results suggest that more attention should be given to the biological effects of petroleum hydrocarbon and PAHs pollution in the JRE and its adjacent waters, and OME-FES can facilitate the application of the multiple biomarker approach in ecological risk assessment and marine biomonitoring.

Acknowledgements

Special thanks are extended to Kuang Weiming and Chen Jinmin from the Third Institute of Oceanography, Ministry of Natural Resources, for their assistance in chemical analysis.

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Supplementary information

Table S1. Concentrations of petroleum hydrocarbons, PAHs, PCBs and OCPs in the artificial seawater (control) and the surface seawater collected from twelve sampling sites in the JRE and its adjacent waters.

Table S2. The primer sequences used in the RT-qPCR analysis.

Fig. S1. Mortality of *O. melastigma* embryos treated with surface seawater samples at different sites compared with the control after 4 d exposure. Data are expressed as the mean ± standard error. Significant differences were accepted at $^*p < 0.05$. Five biological replicates were conducted.

Fig. S2. BSD score of *O. melastigma* embryos treated with surface seawater samples at different sites compared with the control after 4 d exposure. Data are expressed as the mean ± standard error. Significant differences were accepted at $^*p < 0.05$. Five biological replicates were conducted.