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Comparison of toxicity effects of fuel oil treated by different dispersants on marine medaka (*Oryzias melastigma*) embryo

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Abstract

This study aims to evaluate the subacute toxic effects of oil under different treatments on marine organism by simulating natural contaminative processes. In this study, 120# (RMD15) fuel oil was selected as the pollutant and marine medaka (*Oryzias melastigma*) embryos as the experimental organism. The developmental toxicity of different volume concentrations (0.05%, 0.2%, 1% and 5%) of water-accommodated fractions, biologically-enhanced water-accommodated fractions, and chemically-enhanced water-accommodated fractions on the embryos in different exposure time (8, 15 and 22 d) were compared and the content of relevant polycyclic aromatic hydrocarbons (PAHs) was studied (in dispersion and *in vivo*). The subacute toxic effects were assessed in terms of antioxidant activities of enzymes (superoxide dismutase, catalase and glutathione S-transferase) and the blue sac disease (BSD) indexes.The results showed that the BSD indexes of the treatment groups were significantly higher than the respective control groups and showed positive correlations with both concentration and exposure time. The experiments with three antioxidant enzymes indicated that enzymatic activities of the embryos changed dramatically under the oxidation stress of petroleum hydrocarbons, especially after adding the dispersants. With the increase of petroleum hydrocarbon concentration and exposure time, the three enzymes showed different degrees of induction and inhibition effects.

Key words: marine medaka, petroleum hydrocarbon, oil dispersant, antioxidant enzymes, 120# fuel oil

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1 Introduction

In recent years, activities such as petroleum exploration, production, transportation and oil spill accidents have significantly impacted floral and faunal communities, resulting in significant deterioration of coastal and wetland ecosystems (Ko and Day, 2004). Petroleum hydrocarbons can be enriched in marine organisms through food chains to cause sustaining negative impacts on human health ADDIN EN.CITE.DATA (Lee et al., 2010; Pérez-Cadahía et al., 2007). To quickly reduce the impacts of oil spillage in coastal areas, a large number of oil dispersants have been used. Therefore, it is necessary to study the effects of the combined toxicity of oil and the dispersants on marine organisms.

Currently, oil dispersants mainly consist of chemical and biological dispersants. Chemical dispersants can quickly and efficiently accelerate oil from the surface of seawater into the water column (Chapman et al., 2007) to promote diffusion and degradation of oil (Swannell and Daniel, 1999). Due to the use of chemical dispersants, the concentration of aromatic hydrocarbons increases rapidly in the surface layer of seawater ADDIN EN.CITE.DATA (Couillard et al., 2005; Wu et al., 2012). A number of studies have shown that the addition of chemical dispersants can significantly increase the toxicity of oil dispersion ADDIN EN.CITE.DATA (Almeda et al., 2014; Dussauze et al., 2015; González-Doncel et al., 2008; Jung et al., 2012; Wu et al., 2012). It has also been found that chemical dispersants may improve the bioavailability of polycyclic aromatic hydrocarbon (PAHs) with high molecular weights, resulting in the occurrence of lesions in aquatic organisms including fishes (Couillard et al., 2005).

Additionally, the water-soluble fractions of oil can cause abnormal development of fish in the early stages ADDIN EN.CITE.DATA (González-Doncel et al., 2008; Mcintosh et al., 2010). Symptoms of blue sac disease (BSD) include pericardial edema, yolk sac edema, cardiovascular disease, craniofacial anomalies, spinal deformities, and so on ADDIN EN.CITE.DATA (Scott, 2009; Mcintosh et al., 2010; Schein et al., 2009). Many researchers have shown that oil spills have long-term adverse effects on the local marine life in both laboratory and natural conditions ADDIN EN.CITE.DATA (Goodbody-Gringley et al., 2013; Lee et al., 2013; Marques et al., 2014; Peterson et al., 2003). According to statistics, 71 serious oil spill accidents (quantity of a single oil spill > 50 t) occurred in China from 1990 to 2010 and the total oil spill volume reached 22 035 t. In order to contain the accidents, a large number of oil dispersants were sprayed into the sea water. The two dispersants selected in this study were Weipu (WP, biological oil dispersant) and Guangming (GM, chemical oil dispersant). Additionally, the potential long-term environmental effects of dispersants, especially biological dispersants, are largely unknown and worthy of attention and evaluation.

The aim of this study is to understand the joint toxicity effects of two oil dispersants on the early life stages of a marine fish. An ideal marine test organism (Bo et al., 2011), the embryo of marine medaka was selected as the experimental organism to study the toxic effects of petroleum hydrocarbons. In this study, BSD index, the activities of enzymes, mortality, incubation time and

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hatchability were evaluated as indexes of toxicity evaluation. The contents of total petroleum hydrocarbons (TPHs) and PAHs in the test solutions and fish were measured to explore the relationships among toxicity, chemical composition, and the bioconcentration factors of PAHs.

2 Materials and methods

2.1 *Materials*

120# fuel oil (RMD15) and two dispersants (WP and GM) were provided by Dalian Maritime Safety Administration. The 120# fuel oil was produced by China National Petroleum Corporation (Dalian, Liaoning, China). The WP dispersant consists of petroleum-degrading bacteria and nutrient solution. The GM dispersant consists of non-ionic surface active agents and organic solvents. Sea salt was produced by China National Salt Industry Corporation (Tianjin, China). Experimental fishes were reproduced in laboratory conditions for generations, and the parents were provided by Xiamen University (Xiamen, Fujian, China). Main chemical reagents were purchased from Honeywell Corporation (New Jersey, USA) and Merck Corporation (Darmstadt, Germany).

2.2 *Preparation of stock solutions*

The preparation of stock solutions of water-accommodated fractions (WAFs), chemically-enhanced water-accommodated fractions (CE-WAFs, oil + GM), and biologically-enhanced water-accommodated fractions (BE-WAFs, oil + WP) followed the method of Barron and Ka'Aihue ADDIN EN.CITE.DATA (2003) and Singer et al. (2000). According to the method, 25 g oil was added into per liter seawater. After adding the oil, the dispersants were added or not into the seawater in the ratio of 1:5 (dispersant:oil) according to the recommendation of the dispersants manufacturer. The mixtures were mixed for 18 h, and vortex was set at 25% of the total volume when mixing. After standing for 6 h, the bottom aqueous phases were taken out as the stock solutions of WAFs, CE-WAFs and BE-WAFs and stored at 4°C. The contents of PAHs and TPHs were tested daily during the experiment.

2.3 *Fish maintenance*

The marine medaka (*Oryzias melastigma*) tested in this study were maintained in an aquaculture system with the temperature of 26±2°C, the salinity of 30±1‰, and the photoperiod of 14 h: 10 h (light: dark). The fishes were fed with larva of brine shrimp 3 times a day. The aquaculture system renewed one-tenth of the total amount of water daily. The eggs were collected at 11:00–12:00 am and observed by a stereoscope to distinguish the unfertilized eggs, ensuring the eggs used in the experiment could develop into embryos.

2.4 *Experimental design*

In order to eliminate the mutual influence among the embryos, they were placed separately in the 24-well plates (one well for one embryo). To each well was added 2 mL of WAFs (0.05%, 0.2%, 1.0% and 5.0%, v/v), CE-WAFs (0.05%, 0.2%, 1.0% and 5.0%, v/v) or BE-WAFs (0.05%, 0.2%, 1.0% and 5.0%, v/v) for exposure. Seawater was used as a blank control, and 5 g/L of each dispersant (the highest dispersant concentration in CE-WAFs and BE-WAFs) were used as dispersant controls. There were 150 embryos in each treatment group and the exposure period was 22 d (the normal incubation period was about 8 to 10 d). All experimental solutions were prepared freshly and renewed every day. The data of malformation, mortality and hatch were observed and recorded daily and the embryos were removed out of the wells once dead. Each experiment result was repeated 5 times and total number of samples in each treatment was 20. The detailed analytical methods of TPH and PAHs, BSD index and antioxidant defenses were described in 2.5–2.8.

2.5 *TPH and PAHs analyses in test solutions*

The TPH concentrations of WAFs, CE-WAFs and BE-WAFs stock solutions were measured with ultraviolet spectrophotometer before each renewal of solutions. The petroleum hydrocarbons were extracted from WAFs, CE-WAFs and BE-WAFs by n-hexane. Based on the results of full wave scanning in the range of 200 to 300 nm, 225 nm was selected as the best absorption wavelength. The optical densities (OD) at 225 nm of extractants were measured by an ultraviolet spectrophotometer. The values of ODs reflected the TPH concentrations.

The contents of precedence-controlled PAHs, listed by the United States Environmental Protection Agency (USEPA), were measured by a gas chromatograph-mass spectrometer (GC-MS) according to the method described by Wu et al. (2011) and Wang et al. (1994). According to this method, 50 mL stock solutions each of WAFs, CE-WAFs and BE-WAFs were sampled. Each stock solution was extracted by 25 mL of n-hexane. After drying and filtration, the treated n-hexane was concentrated to 1–2 mL by rotary evaporators. Silica gel column chromatography was used to elute the PAHs with 15 mL mixture of n-hexane and dichloromethane (1:1). After concentrating to less than 1 mL, the mixture was transferred to a vial and internal standards (d8-naphthalene, d10-phenanthrene, d12-chrysene, and d12-perylene) were added to the vial. Finally, the mixture was quantified to 1 mL

Extracts were analyzed with an Agilent 6 890 GC coupled with a 5 975 mass selective detector in the selective ion–monitoring mode. The GC was equipped with an Agilent DB-5MS capillary column (30 m × 0.25 mm × 0.25 mm) and its operating conditions were: oven was started at 50°C (held for 2 min), heated to 150°C at a step of 8°C/min, then held for 3 min, and heated to 300°C at 3°C/min with a final hold of 10 min. 1 μL sample was injected at the splitless sampling mode with the inlet at 280°C and the ion source (EI) at 230°C. Nitrogen was used as the carrier gas a flow rate of 1.0 mL/min.

All the quality control procedures satisfied their acceptable ranges and under the optimized conditions, the recoveries of the 16 PAHs were 80–120%. PAH contents of seawater in the aquaculture system were checked and no PAH was detected (below the detection limit of 5 ng/L), thus, ensuring that there was no effect of PAHs in the seawater. The concentrations of TPHs and PAHs were repeated 3 times in each treatment.

2.6 *Malformation assessment*

The teratogenicity of WAFs, CE-WAFs and BE-WAFs to the embryo was assessed using the BSD index. The calculation of BSD referred to the method described by Scott (2009) and Wu et al. (2012). Signs were scored according to the following method: decreased circulation (DC, 0–1), heart malformations (HM, 0–1), yolk sac edema (YE, 0–3), pericardial sac edema (PE, 0–3), loss of activity (LA, 0–1), spinal deformity (SD, 0–1), craniofacial deformity (CD, 0–1), and fin rot (FR, 0–1). In each sign, a higher BSD score meant more serious toxicity effect. The weight for edemas was greater than the other signs because of its closest correlation with mortality and a response range large enough to be classified. The calculation method of BSD index is as follows:

$$BSD = \left[\sum_{d=1}^{n}(DC \cdot E_d) + \sum_{h=1}^{n}(HM \cdot E_h) + \sum_{y=1}^{n}(YE \cdot E_y) + \sum_{p=1}^{n}(PE \cdot E_p) + \sum_{l=1}^{n}(LA \cdot E_l) + \sum_{s=1}^{n}(SD \cdot E_s) + \sum_{c=1}^{n}(CD \cdot E_c) + \sum_{f=1}^{n}(FR \cdot E_f)\right] \Big/ (N \cdot 12), \quad (1)$$

where $\sum_{d=1}^{n} DC \cdot E_d$ is the sum of DC scores of each individual in each treatment group. Similarly, E_d, E_h, E_y, E_p, E_l, E_s, E_c and E_f are the number of individuals showing each symptom, respectively. The highest score of BSD index was 12.

2.7 *Antioxidant defenses*

Enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) were measured in the study. Pretreatment method was as follows: the embryos were rinsed with pre-cooling normal saline to remove any impurities. Surfaces of the embryos were dried with filter paper and about 100 mg sample (50–70 embryos) of each treatment group was weighed. The embryos were transferred to 1.5 mL centrifuge tubes and 9-fold normal saline was added into the tubes. Subsequently, the mixture was homogenized. After that the homogenate was centrifuged for 20 min at the speed of 3 000 r/min (2 012.16 g). The supernatant was extracted and the solution was saved at 4°C.

SOD activity was determined using the hydroxylamine reduction assay of Ōyanagui (1984). In this method, the reduction of hydroxylamine by superoxide anion was monitored at 550 nm. One unit of SOD activity was defined as the amount of enzyme necessary to decrease the reduction of hydroxylamine by 50%. CAT activity was measured at 405 nm by an assay of hydrogen peroxide based on the formation of its stable complex with ammonium molybdate (Góth, 1991). One unit of CAT activity was expressed as kilo units per gram of protein. GST activity was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate (Jakoby, 1985). One unit of GST was defined as the amount of glutathione conjugate formed using 1 mM glutathione and CDNB/min per mg protein. Protein concentration in the tissue samples was determined using a commercially available assay kit.

2.8 *PAHs analyses in vivo*

The pretreatment of PAHs for analyses in vivo was improved according to the method described by Nácher-Mestre et al. (2010).The sampled embryos were freeze-dried in a lyophilizer for 24 h. The embryos (about 0.1 g) were ground to a powder and the samples were weighted. 20 mL NaOH (1 mol/L) was added into the flask and the flask was shaken at a speed of 100 r/min to saponify the powder for 12 h. 20 mL ethanol was added into the flask to acidize the samples for 4 h at 80°C. Subsequently, the samples were extracted three times with 8 mL n-hexane. After desiccation and filtration, the solution was concentrated to 1mL approximately by rotary evaporator at 30°C. The extract was added into a silica gel column that was previously conditioned with 10 mL n-hexane. Then the silica gel column was eluted with 15 mL dichloromethane-n-hexane solution (8:2). Finally, the eluate was concentrated to less than 1 mL by a rotary evaporator. The instrument settings for GC-MS analysis were the same as the previous PAHs analyses for the test solutions. The concentrations of PAHs in vivo were repeated 3 times in each treatment.

2.9 *Statistical analyses*

SPSS 17.0 software was used to analyze the data. All data were tested by Kolmogorov-Smirnov test for normal distribution. For the blue sac disease index, data were demonstrated by the Levene median equal variance test. After Bonferroni post hoc test multiple comparisons, all data were analyzed by one-way analysis of variance, and the significance was determined at $p<0.05$.

3 Results and discussion

3.1 *TPHs and PAHs of stock solutions*

The concentrations of TPHs for WAFs, BE-WAFs, and CE-WAFs stock solutions (100% v/v) was 34.3 ± 2.1 mg/L, 93.6 ± 7.3 mg/L and 135 ± 6.7 mg/L, respectively. In comparison to WAFs, the content of TPHs in BE-WAFs and CE-WAFs was 2.73 fold and 3.94 fold, respectively. Additionally, the content of PAHs of WAFs, BE-WAFs and CE-WAFs were (0.166 ± 0.013) mg/L, (0.387 ± 0.026) mg/L and (0.508 ± 0.086) mg/L, respectively. Compared to the WAFs, the PAH concentrations of BE-WAFs and CE-WAFs increased 2.33 fold and 3.06 fold, respectively.

The content of PAHs for WAFs, BE-WAFs and CE-WAFs are shown in Fig. 1. It shows the 16 PAHs classified according to the priority control as noted by the USEPA. The addition of dispersants increased the total content of PAHs, however, the proportions of PAHs in WAFs, BE-WAFs and CE-WAFs were different. The proportions of low molecular weight PAHs (2–3 rings) in WAFs, BE-WAFs and CE-WAFs were much higher than those of high molecular weight PAHs (4–6 rings). Additionally, the proportion of low molecular weight PAHs was the highest in WAFs, the lowest in CE-WAFs and in between for BE-WAFS. The proportions of high molecular weight PAHs in WAFs, BE-WAFs and CE-WAFs were 18.5%, 31.0% and 35.0%, respectively. The results revealed that the dispersants increase the dispersion efficiency of PAHs and the influence of dispersants on high molecular weight PAHs was more significant than on low molecular weight PAHs. The experimental results were verified by previous research outcomes ADDIN EN.CITE.DATA (Couillard et al., 2005; Wu et al., 2012). Additionally, GM showed higher dispersion efficiency than WP.

3.2 *BSD index*

The BSD index can reflect the teratogenicity of early development of marine medaka. During the 22 d of exposure, as shown in Fig. 2, each treatment could cause teratogenesis of marine medaka during the early stage. The BSD indexes of the treatment groups were higher than the control groups. No changes were observed in the dispersant control groups and the blank control group over time. Additionally, there was no significant difference between the dispersant control groups and the blank control group indicating that the dispersants caused little effect on BSD. It was also observed that the abnormality rates of BSD increased with increasing of concentrations of the experimental groups. The BSD indexes of BE-WAFs and CE-WAFs were significantly higher than those of WAFs and in the highest concentration treatment groups at 22 d, the abnormality rates reached 53.3% ± 10.3% in WAFs, 80.6% ± 12.1% in BE-WAFs and 99.4% ± 16.5% in CE-WAFs. The size order of the BSD indexes was: CE-WAFs > BE-WAFs > WAFs. The no-observed-effect concentrations of WAFs, BE-WAFs and CE-WAFs at 22 d were 0.43%, 0.18% and 0.10%, respectively. As indicated in Fig. 2, the BSD indexes of WAFs, BE-WAFs and CE-WAFs also showed a rising trend with increasing concentrations. Basically, the slopes of concentration–response relationships in higher concentrations were greater than those in lower concentrations of the treatment groups. At different time

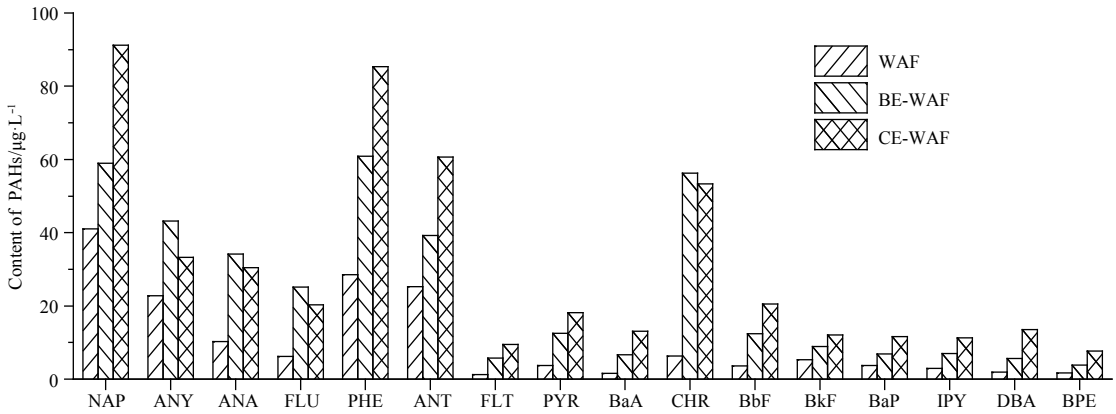

Fig. 1. The concentration of PAHs in initial stock solution of the WAFs, CE-WAFs and BE-WAFs. NAP=naphthalene; ANY=acenaphthylene; ANA =acenaphthene; FLU=fluorene; PHE=phenanthrene; ANT=anthracene; FLT=fluoranthene; PYR=pyrene; BaA=benzo[a]anthracene; CHR=chrysene; BbF=benzo[b]fluoranthene; BkF=benzo[k]fluoranthene; BaP=benzo[a]pyrene; IPY=indeno[1, 2, 3-cd]pyrene; DbA=dibenz[a, h]anthtacene; BPE=benzo[ghi]perylene.

points, the slopes of concentration–response relationships in the treatment groups were different. Over time, the slopes of WAFs, BE-WAFs and CE-WAFs increased from 0.020 to 0.068, 0.038 to 0.163, and 0.056 to 0.171, respectively. This indicated that the longer the exposure time, the greater the BSD indexes would be at the same concentration.

3.3 *Mortality*

The mortalities in all treatment groups are shown in Fig. 3. The mortality of each treatment group increased with an increase of nominal concentrations. The highest mortality (59%) was observed in 5.0% (v/v) CE-WAFs. The mortalities in CE-WAFs were significantly higher than those in BE-WAFs or WAFs ($p < 0.05$) and were 1.5–3 fold those of WAFs at the same concentrations. The mortalities in BE-WAFs and CE-WAFs were higher than those in WAFs at the same concentrations, and the increasing extent of mortality was more remarkable at higher concentrations. It can be observed that the effect of dispersants on the mortalities at high concentrations was greater than that at low concentrations. As shown in Fig. 3, marine medaka embryos were more sensitive to CE-WAFs and BE-WAFs than to WAFs.

3.4 *Hatch*

The effects on incubation period and hatchability of marine medaka by WAFs, BE-WAFs and CE-WAFs are shown in Fig. 4. The results indicated that WAFs, CEWAFs and BE-WAFs can affect the hatch (incubation period and hatchability) of marine medaka. WAFs, BE-WAFs, and CE-WAFs could prolong the incubation time of embryos by different degrees. No significant effects on incubation period were observed in embryos exposed to the control groups. Generally, the incubation time of embryos exposed to WAFs and BE-WAFs increased initially and then decreased with increase in concentration. The incubation period for WAFs and BE-WAFs treatment was most sensitive to the 0.2% concentration. CE-WAFs had the most significant delay effect on the incubation time, however, the differences between concentration gradients were not significant. After exposure to WAFs, BE-WAFs and CE-WAFs, the incubation period was extended from 10–14 d (normal incubation period) to 15–19 d. The mean incubation periods for WAFs, BE-WAFs and CE-WAFs were 15.1 d, 17.0 d and 18.2 d, respectively.

Compared to WAFs, the hatchability of CE-WAFs and BE-WAFs at the same concentration was lower ($p < 0.05$). In fact, WAFs had no significant impact on the hatching rate and the lowest hatchability of 70% was reached at the concentration of 5%. However, the addition of two dispersants had a great influence on the hatching rate of embryos and it was progressively evident with the increasing concentrations. However, individually, the dispersants had little effect on the hatchability.

3.5 *Antioxidant enzyme*

The results indicate significant change of the activities of SOD, CAT and GST in the control groups with the extension of exposure time. In general, the enzyme activity of each treatment group was significantly higher than that of the respective control group. Additionally, the enzyme activities increased over time, but the increase was not significant.

3.5.1 *SOD*

At 8 d exposure, it was found that the SOD activities (Fig. 5) of experimental groups first increased and then decreased with increasing concentrations. Compared to the control groups, SOD activities of experimental groups exhibited significant increase when exposed to all the contaminants. For WAFs, BE-WAFs and CE-WAFs, the highest increase with respect to the SOD activity reached 73%, 78% and 105%, respectively. The peaks appeared at the concentrations of 5.0%, 5.0% and 1.0%, respectively. The SOD activities of WAFs and CE-WAFs showed a significant difference.

Compared to the 8 d values, there was no significant change in the control groups after exposure for 15 d. The SOD activity of each experimental group increased at all concentrations, especially the value of WAFs. The values of BE-WAFs and CE-WAFs first increased and then decreased with increasing concentrations and the peak value of BE-WAFs was higher than that of CE-WAFs. However, the SOD activity for WAFs showed a positive relation with the concentrations. Additionally, the peaks appeared at the concentrations of 5.0%, 1.0% and 1.0% for WAFs, BE-WAFs and CE-WAFs, and were lower than those observed for 8 d.

For the 22 d exposure, the SOD activity of the control groups changed less than before, however, in general, the values of the experimental groups decreased at higher concentrations (1.0%–5.0%). The differences for SOD activity among concentrations reduced during the 22 d of exposure and the peak concentrations got lower than before at 1.0%, 0.2% and 0.2% for WAFs,

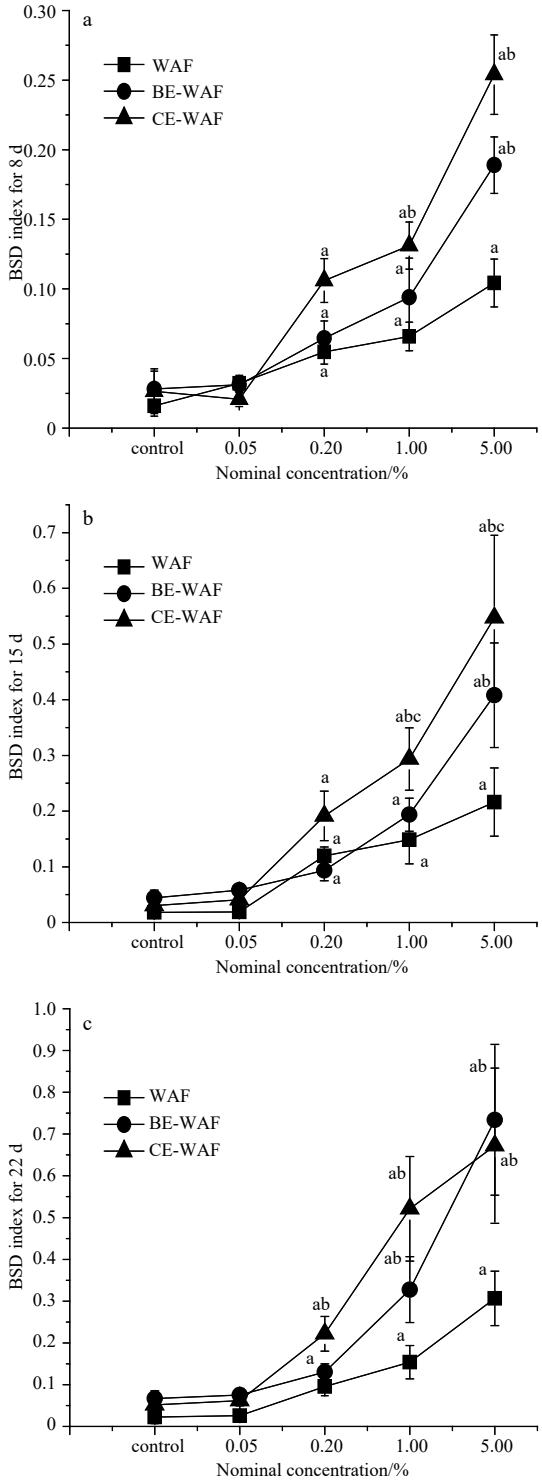

Fig. 2. BSD index in embryos exposed to WAFs, BE-WAFs, CE-WAFs for 8 d (a), 15 d (b) and 22 d (c). The indexes were expressed as mean±standard deviation (n=20 embryos per treatment). "a" indicates that BSD index of WAFs or BE-WAFs or CE-WAFs has a significant difference from the respective control (p<0.05), "b" indicates that BSD index of BE-WAFs or CE-WAFs has a significant difference from that of WAFs at the same concentration (p<0.05), and "c" indicates that BSD index of CE-WAFs has a significant difference from that of BE-WAFs at the same concentration (p<0.05). The same sign is applied to the following figures.

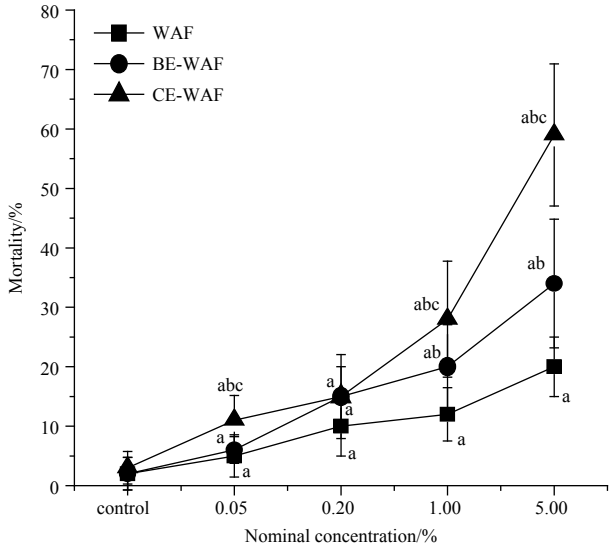

Fig. 3. Mortality of embryos exposed to WAFs, BE-WAFs and CE-WAFs. The mortality means the ratio of the number of dead embryos to the total number of samples. The mortalities were expressed as mean±standard deviation (n=20 embryos per treatment).

BE-WAFs and CE-WAFs, respectively. For the 22 d exposure period, the SOD values of BE-WAFs were the highest among the experimental groups.

3.5.2 *CAT*

CAT activities (Fig. 6) in the embryos of the control groups were at the same level at the end of the 8 d exposure period. CAT activity of each experimental group increased by different degrees with the increase in concentrations. However, the value of CAT decreased at the concentration of 5.0% and the CAT of CE-WAFs showed differences with the other two groups. The induction effect of CE-WAFs was more obvious (the peak concentration was lower and the peak value was higher).

After the 15 d exposure, the results showed that the activities of CAT increased significantly in the treatment groups. The increase in the peak value of each treatment group was significantly higher (p<0.05) than the control group and much higher than those of the 8 d exposure. In general, the exposure concentrations corresponding to CAT peak values of the three experimental groups decreased (1.0% for WAFs and BE-WAFs and 0.2% for CE-WAFs). Furthermore, the increase in CAT activity in the CE-WAFs group was significantly higher than that for WAFs. After the 15 d exposure period, CAT activity remained unchanged for most exposure treatments (when compared to the 8 d exposure levels) at lower concentrations (control and 0.05%). The increase was significantly higher (p< 0.05) than the values for 8 d at higher concentrations (0.2%–5.0%), especially at the concentration of 0.2%. The induction of BE-WAFs and CE-WAFs was significantly greater than WAFs. However, the differences between BE-WAFs and CE-WAFs were not obvious.

At 22 d, the CAT activity of the control groups remained at the same level as before (compared to the levels observed at 8 d and 15 d exposure). However, the CAT values of experimental groups increased by some degree. Some CAT values of BE-WAFs and WAFs at lower concentrations (0.05%–0.2%) increased significantly (p < 0.05) in comparison to the 15 d exposure. Compared to

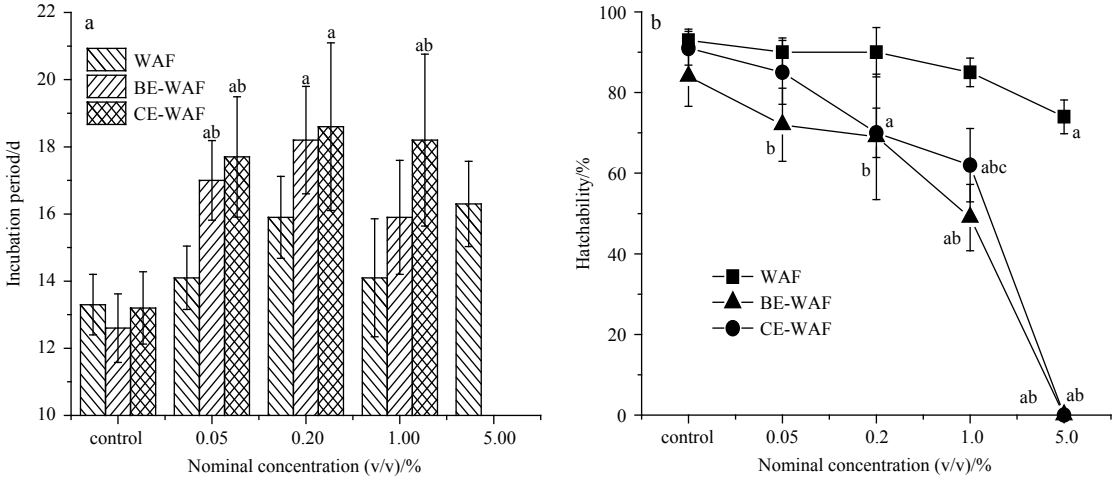

Fig. 4. Incubation period and hatchability of embryos exposed to WAFs, BE-WAFs and CE-WAFs. The hatchability means the ratio of the number of hatched embryos to the total number of samples. The incubation period and hatchability were expressed as mean±standard deviation (n=20 embryos per treatment).

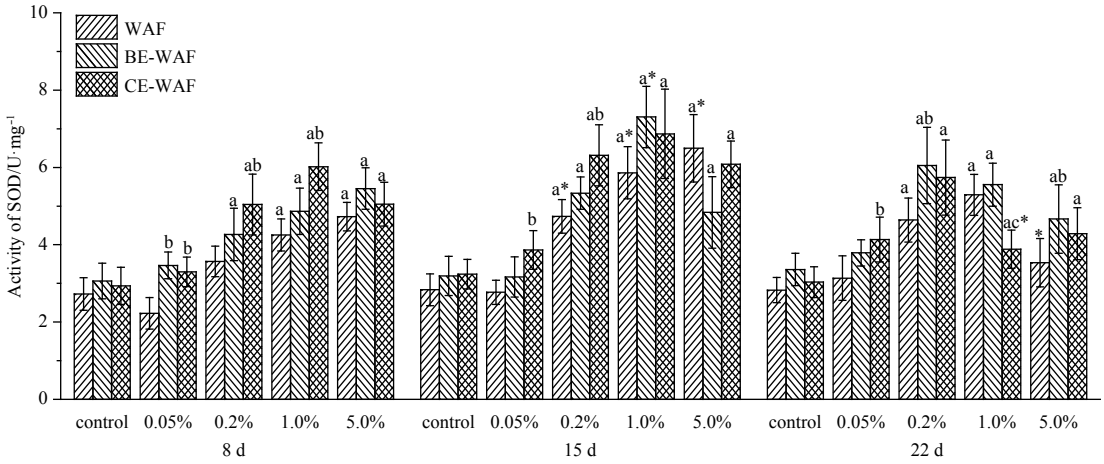

Fig. 5. Superoxide Dismutase (SOD) activity in embryo of medaka. The SOD is expressed as mean±standard deviation (n=10 per treatment). For each exposure treatment, * indicates values significantly different from those of the previous exposure period at the corresponding concentration (p<0.05). The same sign is applied to Figs 6 and 7.

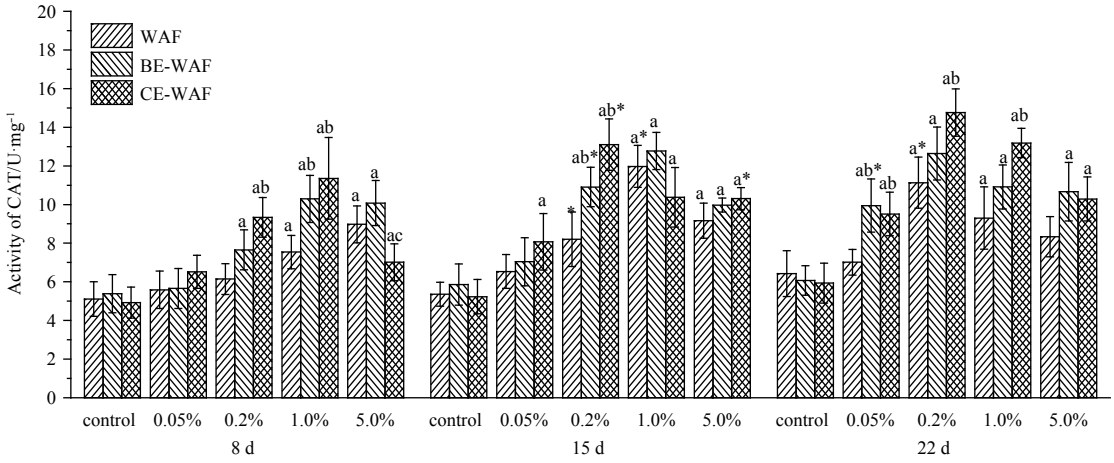

Fig. 6. Catalase (CAT) activity in embryo of medaka.

the 8 d and 15 d, all exposure concentrations corresponding to peak values of experimental groups decreased to 0.20%. The data showed that the CAT values of CE-WAFs were significantly higher than those of WAFs at concentrations ranging from 0.05% to 5.0%. In the 22 d exposure test, it was found that the induction of CAT was more significant than that of 15 d (the peak value of

each treatment group was higher and the corresponding concentration was lower).

3.5.3 *GST*

GST activity (Fig. 7) in the embryos of the control groups was 4.82 ± 0.65, 5.50 ± 1.02 and 4.59 ± 0.69 U/mg of protein for WAFs, BE-EAFs and CE-WAFs respectively after 8 d exposure. The GST activity of each experimental group rose with increase in concentration, however, no significant increase was observed in the embryos exposed to the three experimental groups at lower concentrations. Additionally, the values of the experimental groups peaked at the concentration of 5.0%.

The results after the 15 d exposure indicated that the activities of GST were significantly induced by WAFs, BE-WAFs and CE-WAFs. The GST activity of control groups remained largely unchanged. The GST peak values of WAFs, BE-WAFs and CE-WAFs for 15 d were significantly higher ($p< 0.05$) than those for 8 d. Compared to the values for 8 d, exposure concentrations corresponding to the peak values of the three experimental groups were lower (5.0% for WAFs, 1.0% for BE-WAFs and CE-WAFs). The GST activity of BE-WAFs was significantly higher than that of WAFs at the concentrations of 0.05%, 0.2% and 1.0%.

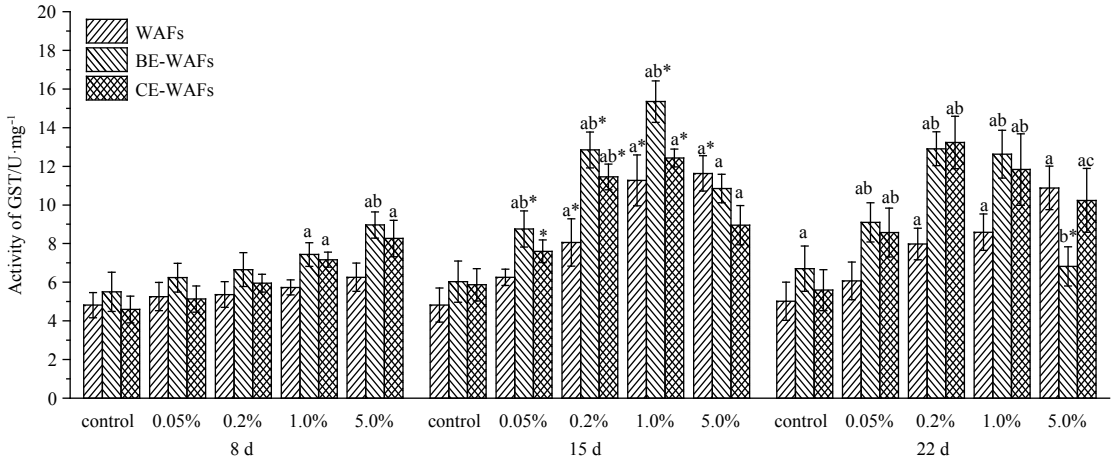

Fig. 7. Glutathione S-transferase (GST) activity in embryo of medaka.

At 22 d, the GST activity of control groups remained at the same level as before (when compared to the levels observed at 8 d and 15 d). However, the values of experimental groups steadily decreased when compared to the values of for 15 d. Additionally, the differences between experimental groups increased than before. The values of BE-WAFs and CE-WAFs were significantly higher than those of WAFs and exposure concentrations corresponding to peak values were different (5.0% for WAFs and 0.2% for BE-WAFs and CE-WAFs).

The effects of WAFs, BE-WAFs and CE-WAFs on antioxidant enzyme activities are shown in Figs 5, 6 and 7. When marine medaka embryos were exposed to WAFs, BE-WAFs and CE-WAFs, the enzyme activities (SOD, CAT, and GST) in vivo changed significantly ($p < 0.05$). As shown in Fig. 5, the treatment groups at lower concentrations could induce enzymatic activities in marine medaka embryos. In WAFs treatment groups, the induction effect of various enzyme activities showed a positive correlation with the concentration. In the BE-WAFs and CE-WAFs treatment groups, the enzyme activities increased first and then decreased with increase in concentration, however, the peaks appeared at different concentrations. Under low concentration exposure, GST reached its peak earlier than SOD and CAT showed more sensitivity. In addition, the GST activity showed some sensitivity in the BE-WAFs control group.

3.6 *PAHs in vivo and dispersions*

The concentrations of PAHs in vivo and dispersions are shown in Table 1. After adding the dispersants, the content of PAHs in the dispersions and the body increased significantly. At the same concentrations, the accumulation of PAHs in CE-WAFs treatment groups was higher than that in BE-WAFs. The PAH concentrations in the embryos exposed to BE-WAFs and CE-WAFs were 1.2–1.9 fold and 2.0–2.6 fold higher, respectively, than those of WAFs at different time points (Table 2). At the early stage of exposure, the PAHs in vivo of all treatment groups increased significantly. However, the PAH concentrations showed no change during the period of 15–22 d.

It is evident from the experimental data seen that the cumulative amounts of PAHs changed with PAH concentrations in dispersions and exposure times. The cumulative amounts of PAHs in vivo were positively correlated to the concentrations of PAHs in the dispersions. The experimental results were consistent with those of Canova (1998) and Skarphéðinsdóttir et al. (2003) on the effect of benzo[a]pyrene on shellfish. The results showed that in the medaka embryos, the PAHs accumulated increasingly rapidly first and reaching a dynamic balance at last. The results correspond to the change process of antioxidant enzymes activities. The antioxidant enzyme activities increased first and then gradually reached stability. The results also indicate that the absorption and metabolism of PAHs in marine medaka embryos gradually reached equilibrium within the range of oil concentrations (0.05%–5.0%) during this experiment.

4 Discussion

In this study, the exposure of 120# fuel oil dispersions to marine medaka embryos caused increased mortality and sublethal effects. The symptoms observed in the experiment were consistent with previous studies of aquatic organisms exposed to various oils ADDIN EN.CITE.DATA (Dubansky et al., 2013; Mcintosh et al., 2010; Wu et al., 2012). This indicated that 120# fuel oil also had similar toxic components. In addition, we also explored the change in antioxidant enzyme (SOD, CAT and GST) activities in the embryos. The toxicity effects on embryos increased significantly after adding dispersants. Generally, the dispersants them-

Table 1. Concentrations of PAHs

Treatment		Summed priority PAHs in marine medaka/ng·g^{-1}			Summed priority PAHs in dispersions/ng·g^{-1}
		8 d	15 d	22 d	
Control	Blank	7.46±0.32	9.31±0.55	6.45±0.59	3.78
	BE	9.76±0.65	11.21±2.09	12.36±0.59	5.23
	CE	13.75±1.47	16.3±2.94	17.49±1.95	7.91
WAFs	0.05%	107.49±21.4	180.99±32.98	216.39±41.13	20.36
	0.2%	160.97±30.31	288.35±50.67	309.11±56.72	39.42
	1.0%	322.96±40.81	499.01±62.71	469.74±58.25	55.94
	5.0%	441.03±42.23	621.52±58.92	637.93±119.24	71.92
BE-WAFs	0.05%	200.78±39.6	305.63±60.02	369.47±63.36	46.38
	0.2%	299.84±52.78	488.31±79.99	467.76±70.25	118.12
	1.0%	600.21±80.38	634.03±73.51	662.27±115.14	170.45
	5.0%	656.23±106.93	705.31±131.74	724.73±136.56	203.47
CE-WAFs	0.05%	251.91±47.95	475.61±75.48	491.45±86.23	76.42
	0.2%	409.46±69.77	626.62±106.78	602.93±116.57	152.61
	1.0%	705.68±111.23	769.37±144.4	732.99±122.8	228.28
	5.0%	818.4±163.89	832.88±142.76	811.64±104.15	263.15

selves were less toxic, however, adding the dispersants could increase the content of TPHs and PAHs in water ADDIN EN.CITE.DATA (Duarte et al., 2010; Lyons et al., 2011) and lead to an increase in toxicity to marine organisms ADDIN EN.CITE.DATA (Carls et al., 1999; Lee et al., 2013). Cohen and Nugegoda (2000) found that the content of TPHs in CE-WAFs was higher than that in WAFs. Therefore, high content of petroleum hydrocarbons could be the main factor affecting the survival and development of marine medaka embryos. Among petroleum hydrocarbons, the PAHs have been observed to cause genotoxicity, carcinogenicity, and reproductive toxicity in marine organisms and are known to be widely distributed in aquatic ecosystems (Bellas and Thor, 2007). Therefore, the contents of PAHs in water and embryos were monitored.

In this experiment, in general, the observed mortality was ordered as CE-WAFs > BE-WAFs > WAFs. The difference in mortalities was associated with the contents of low molecular weight PAHs. Experiments have shown that low molecular weight PAHs (2–3 rings), such as phenanthrene and anthracene, have a nonpolar anesthetic effect on organisms. These PAHs diffuse into the tissues through their hydrophobicity and cause damage to tissue cells ADDIN EN.CITE.DATA (Incardona et al., 2004, 2006; Mu et al., 2012; Schein et al., 2009). The order of 2–3 ringed PAHs content was CE-WAFs (330.7 mg/L) > BE-WAFs (267.47 mg/L) > WAFs (135.32 mg/L). As shown in Fig. 2, BSD indexes of BE-WAFs and CE-WAFs in different periods were significantly higher than those of WAFs ($p < 0.05$). There was a positive correlation between the BSD indexes and the concentration of PAHs (Table 2). The inference that PAHs can induce abnormal development of embryos has been verified in other studies ADDIN EN.CITE.DATA (Billiard et al., 2002; White et al., 1999) as well. In our experiment, similar symptoms, such as loss of moving ability, out of balance, and rotation of body swirled in the newly hatched larvae, were found in the high-concentration groups of CE-WAFs and BE-WAFs. The symptoms were greatly consistent with the description of the effects of petroleum hydrocarbons on the nervous system of fish. Thus, it can be inferred that the addition of dispersant not only increases the content of TPHs and improves its teratogenicity but also enhances the developmental toxicity and neurotoxicity of crude oil on the marine medaka embryos. The symptoms of the two dispersant control groups were similar to those of the seawater control group and almost no PAHs were detected in the control groups. It can be inferred that the dispersants by themselves have little effect on marine medaka embryos' mortality and BSD. Therefore, the high concentration of petroleum hydrocarbons can be ascribed to the peptization of dispersants that makes oil droplets smaller, so that the contact area contacted with water increases, thereby promoting the dissolution of hydrocarbons.

The change in incubation time and hatchability of treatment groups revealed the obstacle to the embryonic development process. In terms of incubation time and hatchability, the higher the concentration of PAHs, the longer the incubation time and the lower the hatchability. It was also found that although these three kinds of dispersions could delay the incubation time, the degree of influence was variable. This result was related to the contents of PAHs. Previous studies have found that PAHs have a significant impact on embryonic development toxicity ADDIN EN.CITE.DATA (Bellas and Thor, 2007; Mu et al., 2012) and thereby, further affect its incubation. In this study, this result was verified in the incubation period experiments. Under lower concentrations (0.05%–0.2%), the addition of dispersants prolonged the incubation period significantly ($p < 0.05$), however, the differences between BE-WAFs and CE-WAFs were not significant.

After exposure to WAFs, the activities of SOD, CAT and GST in marine medaka embryos changed significantly. Activities of the three enzymes increased significantly because of the induction effect of low-concentration petroleum hydrocarbons which are known to increase oxidative stress of ROS in marine medaka embryos. Oxidative stress is known to result in an adaptive induction response in embryos (Livingstone et al., 1990). The induction response was a self-regulatory mechanism to oxidative stress induced by exogenous pollutants (Tissier et al., 2015). Similar conclusions were found in studies on *Liza aurata* (Milinkovitch et al., 2013). In the BE-WAFs and CE-WAFs exposure experiments, the increase in dispersed petroleum hydrocarbons concentration made the induction effect more significant. Additionally, the enzyme activities increased at low concentrations and decreased at higher concentrations (1.0%–5.0%). This phenomenon was due to the continuous oxidative stress of high-concentration petroleum hydrocarbons. The oxidative stress was beyond the defense capacity of the body, thereby, affecting the synthesis of antioxidant enzymes and decreasing the enzyme activities. Cheung et al. (2004) and Richardson et al. (2008) stud-

ied the effects of PAHs on the antioxidant system of mussels (*Emerald mussel*). The results obtained from these studies were similar to the results in this study. Additionally, induction effects of the three enzymes were found to be different. The induction effect of GST was more obvious. In the context of concentration, the peak concentration of GST was lower (0.2%) while the peak concentrations of SOD and CAT were higher (1.0%–5.0%). The results obtained from these studies were similar to those obtained in this study. Our results indicate that GST showed higher sensitivity to low concentration petroleum hydrocarbons in comparison to SOD and CAT. Only GST showed sensitivity to the biological dispersant control group whereas SOD and CAT activities showed no significant changes. This might be due to the presence of degrading microorganisms in the biological dispersant. Moreover, GST has been used as a molecular probe to determine the presence of PAHs degrading bacteria in the studies by Lloyd-Jones and Lau ADDIN EN.CITE.DATA (1997) and Lloyd-Jones et al. (1999).

The results showed that the extent of increase in the bioconcentration factors of PAHs due to the addition of biological and chemical dispersants were 2 fold and 2.4 fold, respectively. This result was consistent with previous studies on dispersed South American crude oil. For example, the addition of the chemical dispersants increased the toxicity of crude oil to mummichog (*Fundulus heteroclitus*) (Couillard et al., 2005) embryos by four times. Ramachandran et al. (2004) found that toxicity of chemical dispersion of South American crude oil to embryos of *Oryzias latipes* in freshwater increased by 56 times. However, the increase in bioconcentration factor and toxicity did not reflect any changes in the toxicity of their constituents. Compared to WAFs, BE-WAFs and CE-WAFs retained more petroleum hydrocarbons in water. As the most toxic constituents in petroleum, the PAH contents of WAFs, CE-WAFs and BE-WAFs showed significant differences. The accumulated PAH contents in embryos were positively correlated with the content of PAHs in water. Moermond et al. (2005) and Thorsen et al. (2004) found similar results in the bioaccumulation experiment on benthos exposed to sedimentary organic pollutants. Therefore, the bioavailability of PAHs in WAFs and BE-WAFs was found to be very close and was lower than that of CE-WAFs. However, the bioavailability was at the same level for all control groups. In comparison to WAFs, the addition of chemical dispersant increased the joint toxicity of CE-WAFs. The negative effects of biological dispersant were not observed in this experiment.

5 Conclusions

In this study, joint toxicities of different dispersants and 120# fuel oil on marine medaka (*Oryzias melastigma*) embryos were compared and the relationships between toxicities and petroleum hydrocarbons contents, especially the contents of PAHs, were explored. The results indicate that the use of dispersant increases the content of TPHs and PAHs in water and changes the relative proportion of PAHs, thus, increasing the damage to marine medaka embryos. In order to determine the physiological indexes, change of development deformity, mortality rate, and enzyme activity were observed after adding the dispersants. With respect to petroleum hydrocarbons content, it was found that the addition of dispersants increased the bioconcentration factors of PAHs. The results demonstrate that chemical and biological dispersants can improve the content of TPHs and PAHs in water. The content of PAHs in BE-WAFs, especially the content of high molecular weight PAHs, was lower than that of CE-WAFs. The toxic effects of BE-WAFs, such as mortality, blue sac disease, and enzyme activity were lower than those observed in CE-WAFs but higher than those in WAFs. Thus, compared to chemical dispersants, the biological dispersant can reduce the toxic effects of crude oil. However, compared to crude oil, the biological dispersant can cause more obvious biological toxicity and lead to potential long-term impacts on marine ecosystems.

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