The PAH characterization and the acute toxicity of diesel oil and chemically dispersed diesel oil to *Litopenaeus vannamei*

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Abstract. Oil spills are a major contributor to marine pollution. It can threaten marine ecosystem function and cause significant damage over both a short and long time period. The aims of this study were to quantify and characterize the polycyclic aromatic hydrocarbon (PAH) of diesel oil water accommodated fraction (WAF) and chemically enhanced WAF (CEWAF), and to determine the LC_{50} of diesel oil WAF and CEWAF at both pH 6.5 and 8.5 to *Litopenaeus vannamei*. PAH quantification and characterization were using high-performance liquid chromatography method, while the LC_{50} determination was using acute toxicity test. Diesel oil WAF was much less toxic than that of diesel oil CEWAF, in which the LC_{50} values of the WAF were 177.25 g L^{-1} and 205.42 g L^{-1} for pH 6.5 and 8.5 respectively, while the LC_{50} values of the CEWAF were 39.59 g L^{-1} and 72.15 g L^{-1} for pH 6.5 and 8.5 respectively. The WAF contained lower concentration of PAH than the CEWAF (0.84 mg L^{-1} and 3.16 mgL^{-1} PAH respectively), in which anthracene was the most dominant type of PAH. Those with the higher amount of PAH in CEWAF led the higher toxicity to *L. vannamei*. However, the addition of chemical dispersant to the diesel oil WAF does not cause a synergistic toxicity effect to the whiteleg shrimps, *L. vannamei*.

Key Words: *Litopenaeus vannamei*, diesel oil WAF, CEWAF, PAH, acute toxicity test.

Introduction. Oil spills into the marine ecosystem can have devastating consequences, environmentally as well as economically and socially. The spills may be due to releases of crude oil from offshore platforms, oil tankers, and offshore drilling rigs and wells, as well as petroleum refinery products such as asphalt base, gasoline, and diesel oil (Mishra & Kumar 2015; Asadi et al 2017a). In late March 2018, an oil spill accident in Balikpapan port city, Indonesia caused a fire, leading to the deaths of five fishermen and causing hundreds of local people to become sick. The spills covered 120 km^2 of Balikpapan bay spreading out by waves and currents, damaging its marine ecosystem and directly killing marine organisms in the affected area. The spills even killed the endangered Irrawaddy dolphin (*Orcaella brevirostris*) on the coast shortly after the disastrous spills (Kahfi 2018; McKirdy 2018).

During the Deepwater Horizon oil spill in the northern Gulf of Mexico in 2010, nearly 2 million gallons of chemical dispersants were applied over three months at both the discharging wellhead in approximately 5,000 feet of water and the sea surface (Almeda et al 2014; Seidel et al 2016). The dispersant is the mixtures of solvents and emulsifiers, along with other compounds that reduce the interfacial tension between water and oil. It helps the breakup of oil slicks into small droplets that disperse throughout a water column. The application of oil dispersants does not reduce the toxicity of oil but increases the amount of PAH in the water column (Lyons et al 2011; Wise & Wise Sr. 2011). The oil dispersant itself is an unpleasant chemical concoction which contains a high amount of PAHs that are toxic to organisms (Ramachandran et al 2004).

The application of chemical dispersants is intended to protect the coastal ecosystem by preventing oil slicks coming ashore. However, the oil dispersant use involves a trade-off between exposing marine life on the surface (e.g. mangroves, seabirds) and animals dwelling underwater (e.g. fish, corals, oysters), in which the dispersed oil hugely increases the exposure of wildlife to the toxic PAHs (Goodbody-Gringley et al 2013).

The effects of oil and dispersed oil to marine organisms vary depending on the exposed species, methodologies used in the experiments, and PAHs characteristics of the oil and dispersant (Singer et al 2001; Huang et al 2011; Holth et al 2014; Asadi & Khoiruddin 2017). Water-accommodated fraction (WAF) and chemically-enhanced WAF (CEWAF) should be prepared for laboratory toxicity testing that are more representative of field conditions and represent compounds with high bio-availability towards the test organisms (Singer et al 2001).

In this experiment, the whiteleg shrimps, *Litopenaeus vannamei* were exposed to WAF and CEWAF of diesel oil with pH 6.5 and 8.5. This species lives inshore in tropical marine habitats feeding on bivalves, worms, crustaceans, and benthic detritus at the bottom of the sea (Martínez-Córdova & Peña-Messina 2005), in which as a benthic organism, *L. vannamei* is prone to dispersed oil pollution in the sediment and water column. This species is a crustacean that is also regularly molting in order to grow, elevating its vulnerability toward oil and oil dispersant chemical substances.

Material and Method

***L. vannamei* and culture condition**. Early post-larvae (6 days old) *L. vannamei* were provided by PT Central Pertiwi Bahari, Situbondo, Indonesia, and were kept in the hydrology laboratory, Brawijaya University. The shrimps were maintained in a 120 cm x 60 cm glass aquarium tank with seawater taken from Lenggoksono waters, East Java. The tank was aerated and adjusted at pH 7.5±0.1, salinity 21±0.5‰, and temperature 25±1^{o}C. The shrimps were fed with brine shrimps during the early development until the age of 9 days old. Prior to the experiment, the shrimps were fed with formulated feed with a minimum 35% protein and 30% fishmeal (Subramani & Michael 2017). Approximately a quarter of seawater was replaced daily with fresh natural seawater in order to reduce the nitrate and ammonia content in the tank.

Test chemicals, WAF & CEWAF preparation, and PAHs characterization. pH adjustments were performed by adding HCL and NaOH solution in order to decrease and increase the pH level respectively. pH 6.5 and 8.5 were chosen for the experiment as the values were still in the survival range of *L. vannamei*. Therefore, the pH would not interfere with the results of the experiment in which *L. vannamei* have been known to have a 100% survival rate under the pH levels between 5 and 9 (Liu et al 2009; Furtado et al 2015). Meanwhile, the oil spill dispersant used in this experiment was a commercially used dispersant with a high efficiency to break oil in both aqueous and non-aqueous systems.

WAF used in this experiment was the extract or the aqueous phase of diesel oil which is naturally released from oil in contact with water. The oil was a commercial diesel oil purchased from a PERTAMINA petrol station that is also commonly used by local fishermen to operate the fishing boats' engine. The oil was used to prepare WAF and CEWAF solution according to Chemical Response to Oil Spills-Ecological Effect Research Forum (CROSERF) guidelines with minor modifications (Singer et al 2001). To prepare WAF, 160 mL of diesel oil was diluted with 1600 mL of natural seawater (salinity 21‰) in a sealed 2-L aspirator glass bottle with detachable transparent stopcock. The mixture was then mixed in the dark using a magnetic stirrer with low energy mixing (no vortex, approximately 200 rpm) to minimize degradation and volatilization of the petroleum components.

To prepare CEWAF, 160 mL of diesel oil and 8 mL of chemical dispersant were mixed with 1600 mL seawater using the same aspirator glass bottle. In CEWAF preparation, 25% of vortex was initiated in the water mass first, then dispersant and diesel oil were both poured directly into the center of vortex consecutively. Furthermore, after being stirred for 20-24 hours, the mixtures of both WAF and CEWAF were left to settle for 1 hour. The aqueous phases of both mixtures were then drawn from the stopcock of the aspirator bottle and transferred into flasks. The WAF and CEWAF solution were then used immediately for the subsequent experiments (Almeda et al 2014; Asadi et al 2017a).

The quantification and qualification of PAH in both WAF and CEWAF were analyzed using the high-performance liquid chromatography (HPLC) technique performed at the organic chemistry laboratory, Indonesian Institute of Science (LIPI), Jakarta. This method was chosen as it is reliable, quick and sensitive even with a low quantity of samples. This method could also analyze the hydrocarbon component simultaneously, resulting in many types of hydrocarbons and their quantities in a single sample examination (Gilbert 1987).

Acute toxicity test. Standard static 72-h acute toxicity tests were performed to evaluate the short-term influence of acidic and basic environment on diesel oil WAF and CEWAF using the procedures described by Lee et al (2013). The whiteleg shrimps, *L. vannamei* have been known to survive even at 100% of crude oil WAF concentration (Asadi & Khoiruddin 2017). As diesel oil WAF is less toxic than crude oil WAF (Almeda et al 2014; Asadi et al 2017a; Holth et al 2014), the maximum diesel oil WAF concentration used in this experiment was also 100%. Meanwhile, the maximum diesel oil CEWAF concentration used in this experiment was 32%. This is due to the fact that dispersant alone was much more toxic than any type of WAF (Asadi et al 2017a), and 24% of crude oil CEWAF only killed 80% of *L. vannamei* at the longest time exposure (Asadi 2018).

This experiment was performed in a triplicate. Briefly, 12 shrimps were kept in 1-L Erlenmeyer flasks containing natural seawater with salinity of 21±1 ‰ and temperature of 25±1°C. The diesel oil WAF concentrations for this acute toxicity test were 0%, 25%, 50%, 75%, and 100%, while the diesel oil CEWAF concentrations were 0%, 4%, 8%, 16%, and 32%. The experiments were performed at pH values of 6.5 and 8.5, in which a total of 60 Erlenmeyer bottles were set up for the diesel oil WAF and CEWAF experiments. Furthermore, the observations of water qualities and shrimp mortalities were performed at 6, 12, 24, 36, 48, and 72 hours. The data of mortalities were then plotted and the LC_{50} values were interpolated from the graphs.

Data analysis. Both the experiment and data analysis were performed between June-September 2017. The mortality data was calculated using Microsoft Excel and analyzed using two-way ANOVA followed by Tukey's range test. The percentages of mortality data after control correction were used to transform the mortality data to probits, and the 72-h LC_{50} values of WAF and CEWAF at both pH 6.5 and 8.5 were obtained from the equations of the probit curves as function of log concentration. The 72-h LC_{50} values were then analyzed using two-way ANOVA to determine the significant differences of those data.

Result and Discussion

Water quality parameters. Temperature, salinity, and pH values of diesel oil WAF and CEWAF at both pH 6.5 and 8.5 were measured regularly at 6, 12, 24, 36, 48, and 72 hours. The observations showed that the water qualities of WAF and CEWAF at both pH 6.5 and 8.5 did not change significantly in the course of experiments (Tables 1 and 2).

Table 1

The water quality parameters of diesel oil WAF, presented as the mean±standard error at 6, 12, 24, 36, 48, and 72 hours observation

Concentration	*WAF pH 6.5*			*WAF pH 8.5*		
	Salinity (‰)	*Temperature (°C)*	*pH*	*Salinity (‰)*	*Temperature (°C)*	*pH*
0% (Control)	21±0.0	25±0.7	6.5±0.2	21±0.4	25±0.2	8.6±0.1
25%	21±0.5	26±0.5	6.6±0.1	20±0.5	26±0.0	8.5±0.5
50%	21±0.5	26±0.5	6.5±0.2	20±0.4	26±0.2	8.5±0.1
75%	20±0.5	26±0.5	6.6±0.2	21±0.5	26±0.5	8.5±0.5
100%	21±0.4	25±0.5	6.5±0.1	20±0.4	25±0.4	8.4±0.1

Table 2

The water quality parameters of diesel oil CEWAF, presented as the mean±standard error at 6, 12, 24, 36, 48, and 72 hours observation

Concentration	*CEWAF pH 6.5*			*CEWAF pH 8.5*		
	Salinity (‰)	*Temperature (°C)*	*pH*	*Salinity (‰)*	*Temperature (°C)*	*pH*
0% (Control)	21±0.5	26±0.5	6.6±0.5	21±0.4	26±0.2	8.6±0.1
4%	21±0.0	26±0.5	6.4±0.2	20±0.4	26±0.5	8.5±0.5
8%	20±0.5	26±0.5	6.5±0.2	21±0.4	26±0.5	8.4±0.5
16%	21±0.4	25±0.7	6.5±0.2	21±0.5	26±0.5	8.5±0.5
32%	20±0.0	25±0.5	6.5±0.1	21±0.4	26±0.4	8.5±0.5

PAHs of WAF and CEWAF quantification and characterization. HPLC analysis showed that diesel oil WAF contained 9 types of PAH with total concentration of 0.84 mg L^{-1}. On the other hand, CEWAF contained only 8 types of PAH but it had much higher PAH concentration (3.16 mg L^{-1}). The carbon of the organic compounds ranged from C_{10} to C_{18} in which Anthracene had highest concentration in both WAF and CEWAF (0.25 and 1.77 mg L^{-1} respectively). Meanwhile, 2-Bromonaphtalene ($C_{10}H_7Br$) was only found in CEWAF with a relatively high concentration (0.54 mg L^{-1}). The PAH types and their concentration in both WAF and CEWAF are presented in Table 3.

Table 3

PAHs of diesel oil WAF and CEWAF

PAH	*Molecular formula*	*WAF (mg L^{-1})*	*CEWAF (mg L^{-1})*
Naphthalene	$C_{10}H_8$	0.2131	0.0158
Acenaphthylene	$C_{12}H_8$	0.0112	NF
2-Bromonaphtalene	$C_{10}H_7Br$	NF	0.5459
Acenaphthene	$C_{12}H_{10}$	0.0478	NF
Fluorene	$C_{13}H_{10}$	0.2334	0.2458
Anthracene	$C_{14}H_{10}$	0.2585	1.7773
Phenanthrene	$C_{14}H_{10}$	0.0621	0.1350
Fluoranthene	$C_{16}H_{10}$	0.0050	0.0877
Pyrene	$C_{16}H_{10}$	0.0045	0.2394
Chrysene	$C_{18}H_{12}$	0.0054	0.1166
Total		0.8411	3.1636

Note: NF = Not Found. Units: mg L^{-1} (ppm).

Toxic symptoms, mortality, probit analysis, and the LC_{50} of both WAF and CEWAF. The indications of adverse effects produced by the PAH of WAF and CEWAF could be observed from the decrease of the test animal's activities and the gradual loss of the body balance. In a higher concentration and longer time exposure, those toxicants led to comatose followed by the death of the test animals. Brown flocks were also observed in the thorax of the shrimps, in which those manifestations of toxic effects were very obvious in the highest WAF and CEWAF concentration.

Two-Way ANOVA analysis of the mortality data showed that pH values did not significantly cause the mortality of the animals (p = 0.65). Only the chemical compounds concentrations significantly altered the mortality of the shrimps (p = 0.001), in which the higher concentration resulted in the higher mortalities of the shrimps (Table 4).

Table 4

Two-Way ANOVA analysis of the mortalities of *L. vannamei* exposed to WAF and CEWAF with both pH 6.5 and 8.5

Source	*DF*	*Adj SS*	*Adj MS*	*F-value*	*P-value*
Factor (WAF & CEWAF with different pH)	5	24.83	4.966	2.19	0.065
WAF & CEWAF concentration	8	570.23	71.279	31.37	<0.001
Error	76	172.70	2.272		
Lack-of-Fit	16	29.37	1.835	0.77	0.713
Pure Error	60	143.33	2.389		
Total	89	1131.16			

Note: DF = degree of freedom, Adj SS = the adjusted sum of squares, Adj MS = the adjusted mean of squares, F-value = variance of the group means, P-value = probability value.

As the Two-Way ANOVA analysis of all mortality data revealed that pH values did not significantly cause the mortality of *L. vannamei, a* post hoc test using Tukey analysis was then computed to reveal which chemical that was significantly influenced by pH differences. It revealed that the mortality at WAF with pH 6.5 was significantly different than that of pH 8.5. On average WAF at pH 6.5 led to 5.33 individual mortality of the shrimps, while WAF at pH 8.5 only killed 3.73 shrimps. Meanwhile, the mortality of the shrimps at CEWAF with pH 6.5 did not significantly differ to that of pH 8.5 (Table 5).

Table 5

Tukey analysis of the mortalities with different pH values

Source	*N (flasks)*	*Mean* (individual)
WAF 6.5	15	5.33
WAF 8.5	15	3.73
CEWAF 6.5	15	9.06
CEWAF 8.5	15	8.80

Furthermore, the 72-h mortality probit curves showed that log concentration of WAF and CEWAF at both pH 6.5 and 8.5 had very high correlation with the probit values. The R^2 for WAF were 0.994 and 0.952 for pH 6.5 and 8.5 respectively; meanwhile, the R^2 for CEWAF were 0.982 and 0.991 for pH 6.5 and 8.5 respectively. The equations resulting from the linear regression of probit curves as function of log concentrations were then used to calculate the LC_{50} of WAF and CEWAF at both pH 6.5 and 8.5 (Figures 1 and 2).

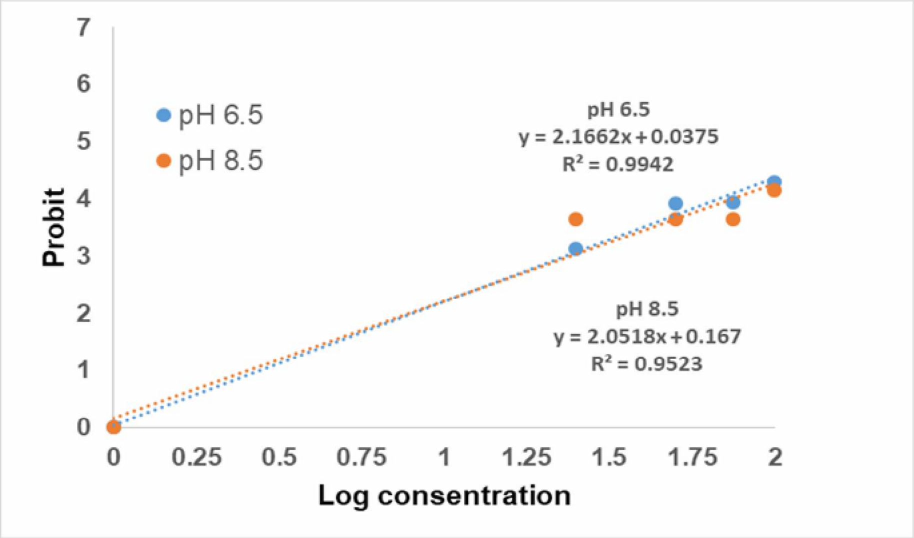

Figure 1. The 72-h mortality probit curves of *L. vannamei* as function of log concentration of WAF at both pH 6.5 and 8.5.

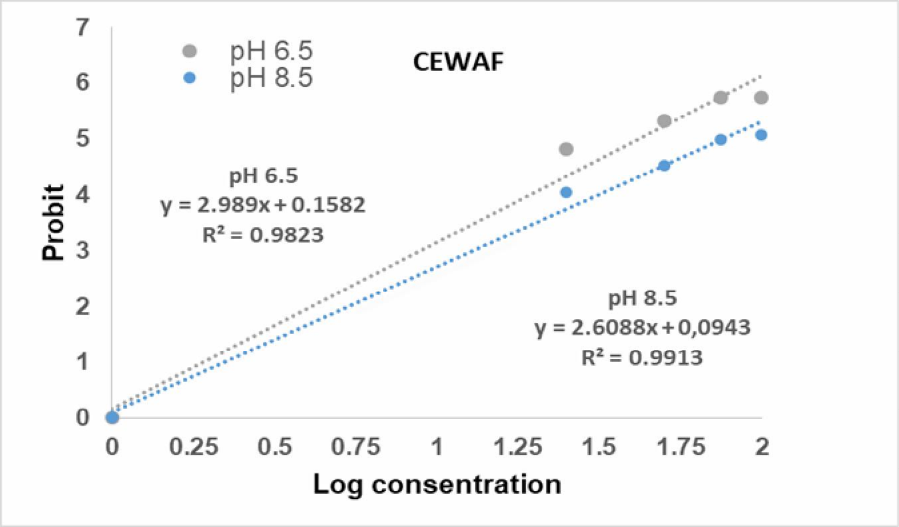

Figure 2. The 72-h mortality probit curves of *L. vannamei* as function of log concentration of CEWAF at both pH 6.5 and 8.5.

Based on the calculation of linear regression equations of the probit models, the 72-h LC_{50} values of *L. vannamei* exposed to WAF were significantly different than that of CEWAF. In WAF and CEWAF, the LC_{50} values of pH 6.5 were also significantly different than that of pH 8.5 (Table 6). The LC_{50} values of WAF were 177.25 g L^{-1} and 205.42 g L^{-1} for pH 6.5 and 8.5 respectively, while the LC_{50} values of CEWAF were 39.59 g L^{-1} and 72.15 g L^{-1} for pH 6.5 and 8.5 respectively (Figure 3).

Table 6

Two Way ANOVA analysis of the 72-h LC_{50} values *L. vannamei* exposed WAF and CEWAF at both pH 6.5 and 8.6

ANOVA table	*SS*	*DF*	*MS*	*F (DFn, DFd)*	*P value*
Interaction	14.36	1	14.36	F (1, 8) = +infinity	$p < 0.0001$
Row factor	55057	1	55057	F (1, 8) = +infinity	$p < 0.0001$
Column factor	2766	1	2766	F (1, 8) = +infinity	$p < 0.0001$
Residual	0	8	0		

Note: DF = degree of freedom, SS = sum of squares, MS = mean of squares, P-value = probability value.

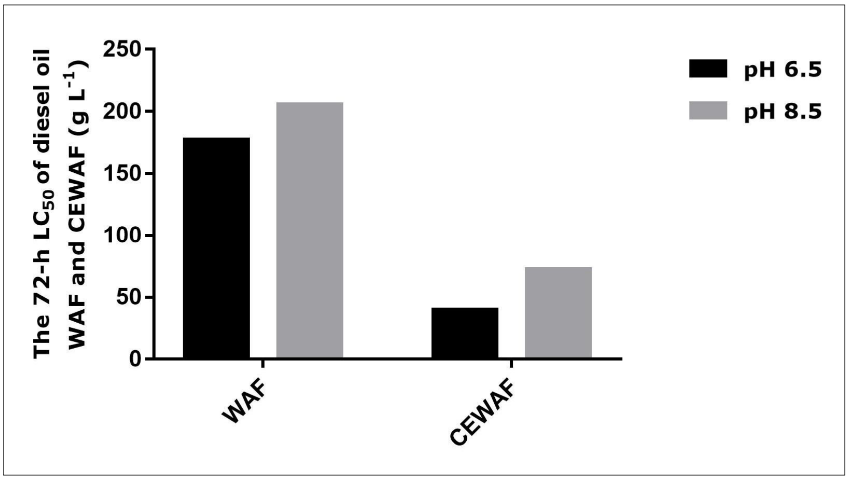

Figure 3. The 72-h LC_{50} of *L. vannamei* exposed to WAF and CEWAF at pH 6.5 and 8.5.

Discussion. WAF and CEWAF of diesel oil were used to evaluate the acute toxicity of dieasel oil and chemically dispersed diesel oil to *L. vannamei* under the acidic and basic environment, in which the chemical concentrations and the test durations were suitable to the species being tested. The water quality parameters of the experimental setup did not have a negative impact on the test animals. The shrimps normally live in tropical marine environment with a temperature higher than 20^{o}C (Kibenge 2016), in which the temperatures in this experiment were between 25^{o}C and 26^{o}C (Tables 1 and 2). This species is also an euryhaline organism that is tolerant to a wide fluctuation in the salinity of water (Chong-Robles et al 2014), in which the optimal salinity regime for its survival is at 20 to 30 ‰ (Amrillah et al 2015; Asadi & Khoiruddin 2017). The shrimps were also reported to survive in a wide range of pH between 5 and 9 (Furtado et al 2015). Therefore, in this experiment, pH values alone would not affect the mortality of the shrimps that could interfere the results of this study.

Furthermore, the PAHs quantification and qualification of WAF and CEWAF showed that there were 9 and 8 types of PAH in the diesel oil WAF and CEWAF respectively. This diesel oil WAF had much lower PAH concentration and fewer number of PAH types than that of crude oil WAF, in which it contains as much as 8.71 mg L^{-1} PAH and as many as 14 types of PAH (Asadi & Khoiruddin 2017), while the PAH concentration of the diesel oil WAF in this experiment was only 0.84 mg L^{-1} (Table 3). The fewer PAH types and the lower PAH concentration in the diesel oil WAF resulted the higher LC$_{50}$ values, 177.25 gL^{-1} and 205.42 g L^{-1} for pH 6.5 and 8.5 respectively (Figure 3). It can be concluded that diesel oil WAF is less toxic than crude oil WAF as the LC$_{50}$ values of crude oil WAF are also much lower, 101.7 g L^{-1} and 114.6 g L^{-1} for pH 6.5 and 8.5 respectively (Asadi & Khoiruddin 2017).

The application of dispersant to break up oil slicks elevates the amount of chemical compound in the environment (Goodbody-Gringley et al 2013). The HPLC analysis showed that diesel oil CEWAF contained 3.16 mg L^{-1} PAH which was more than triple as much as PAH concentration in the diesel oil WAF (Table 3). Therefore, the diesel oil CEWAF also resulted in much higher toxicity than that of diesel oil WAF expressed in the lower LC$_{50}$ values (39.59 g L^{-1} and 72.15 g L^{-1} for pH 6.5 and 8.5 respectively). Meanwhile, the crude oil CEWAF is much more toxic than that of diesel oil CEWAF with much lower LC$_{50}$ values, 8.69 g L^{-1} and 9.29 g L^{-1} for pH 6.5 and 8.5 respectively. It can be easily understood as the PAH concentration of crude oil CEWAF is up to 6-fold higher than that of diesel oil CEWAF (Asadi 2018).

Anthracene ($C_{14}H_{10}$) was the most dominant in both diesel oil WAF and CEWAF with concentration of 0.25 mg L^{-1} and 1.77 mg L^{-1} respectively (Table 3). In marine phytoplankton, *Tetraselmis chuii,* 3.32 mg L^{-1} of anthracene could result in 50% mortality in the species. The anthracene concentration in both diesel oil WAF and CEWAF was a millions times higher than that which naturally occurred in the marine environment, which is only 67 pg L^{-1} (Nizzetto et al 2008).

Furthermore, based on the two-way ANOVA analysis of the LC$_{50}$ of both diesel oil WAF and CEWAF, acidic environment (pH 6.5) significantly elevated the toxicity of both WAF and CEWAF to the *L. vannamei*. However, in comparison with the basic environment (pH 8.5), the diesel oil WAF and CEWAF in acidic environment did not cause synergic toxicity effect to the test animals as the effect of pH 6.5 in diesel oil WAF and CEWAF were less than double than that of pH 8.5. Moreover, the mixture of diesel oil and dispersant do not cause synergistic toxicity to *L. vannamei* as the LC$_{50}$ values of chemical dispersant were lower between 0.19 and 0.55 g L^{-1} (Asadi et al 2017b), suggesting that the chemical dispersant might be sequestered by oil and not present at toxic concentrations (Adams et al 2014). The sequestration mechanisms were reflected in acenaphthylene and acenaphthene, in which those PAHs only presented diesel oil WAF and were not found even in a trace amount in the diesel oil CEWAF (Table 3).

Conclusions. This study demonstrates that chemically dispersed diesel oil is more toxic than diesel oil alone to *L. vannamei*. It is represented by the lower LC$_{50}$ values of diesel oil CEWAF (39.59 g L^{-1} and 72.15 g L^{-1} for pH 6.5 and 8.5 respectively), than those of diesel oil WAF (177.25 g L^{-1} and 205.42 g L^{-1} for pH 6.5 and 8.5 respectively). The higher toxicity of diesel oil CEWAF is due to the fact that it has higher PAH concentration than diesel oil WAF, in which diesel oil CEWAF contains 3.16 mg L^{-1} of PAHs which is more than 3-fold higher than that of diesel oil WAF (0.84 mg L^{-1} of PAHs). Anthracene constitutes 30% and 56% of PAH in diesel oil WAF and CEWAF respectively. Therefore, this chemical compound may have high contribution in the toxicity of those WAF and CEWAF to *L. vannamei*. Although diesel oil CEWAF is more toxic than diesel oil WAF, and the WAF or CEWAF at pH 6.5 was also more toxic than that of at pH 8.5, there is no synergistic toxicity confirmed in this experiment. This may be due to the sequestration mechanism of dispersant by oil once it is mixed in the environment.

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