pH effects in the acute toxicity study of the crude oil-WAF (water accommodated fraction) in the whiteleg shrimp, *Litopenaeus vannamei*

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Abstract. Oil spillage can cause harmful risks to marine ecology in a short time period and may lead to devastating long-term impacts. Meanwhile, the trends of a pH decrease due to ocean acidification deteriorate spillages' impact. This study evaluated the influence of pH on crude oil water accommodated fraction (WAF) toxicity to the whiteleg shrimp, *Litopenaeus vannamei*. Post larvae of the shrimps were exposed to the crude oil-WAF with concentrations of 0%, 25%, 50%, 75%, and 100% under pH concentrations of 6.5 and 8.5 for 72 hours to quantify their mortality. The polycyclic aromatic hydrocarbons (PAHs) of the WAF were analyzed using the GC-MS method, while the LC_{50} was determined using probit analysis. *L. vannamei* showed impaired swimming ability, loss of balance, comatose, and even death when the shrimp were acutely exposed to the crude oil WAF. The 72-h LC_{50} were slightly lower in pH 6.5 than that of 8.5 (101.7 ± 9.6 mL L^{-1} and 114.67 ± 11.7 mL L^{-1} respectively). There were 14 PAH compounds presented in the crude oil-WAF in which carcinogenic compound, benzo[a]pyrene, represented 25% of the total concentration of PAHs. The interaction among PAHs may lead synergistic effects that could increase the mortality of the shrimps. However, based on the US EPA's LC_{50} scale, the crude oil-WAF is still practically non-toxic to the whiteleg shrimp, *L. vannamei*.
Key Words: *Litopenaeus vannamei*, crude oil WAF, acute toxicity test, LC_{50}, benzo[a]pyrene.

Introduction. With the increasing global energy demand, offshore production and transportation of petroleum hydrocarbon is steadily increasing, leading to more catastrophic oil spills in the ocean, e.g. Deepwater horizon (DWH), Bohai Bay accidents, and Sundarbans oil spill in 2010, 2011, and 2014 respectively (Jiang et al 2012; Bautista & Rahman 2016). In DWH oil spills, the total discharge of crude oil in the Gulf Mexico environment was 4.9 million barrels, which is considered as the largest marine oil spill in the history of the petroleum industry. The spill was due to the explosion of a drilling rig in a deep exploratory well (Ali et al 2014); meanwhile, the Sundarbans oil spill occurred after an oil-tanker named Southern Star VII collided with a cargo vessel, spreading and affecting an area of over 140 sq. mi of a UNESCO World Heritage site delta (Bautista & Rahman 2016).

The increasing discharges of crude oil and refined oil into sea progressively pose risks to marine ecosystems. The spills immediate affect and have long-term potential impact on populations of marine organisms. The adverse effect of spills in a short period of time, whether from a single exposure or from multiple exposure, is described as acute toxicity, whereas a longer period of exposure to marine organisms results in chronic toxicity (Jiang et al 2012; Holth et al 2014).

When spilled, the various types of petroleum hydrocarbon can affect the environment differently due to differing from each other in their toxicity, composition, viscosity, and solubility (Huang et al 2011; Rico-Martínez et al 2013; Holth et al 2014). Moreover, crude oils from different exploratory wells within the same geographic area may differ qualitatively in their chemical characteristics (Zhan et al 2015). Besides this, the toxicity effects of crude oils also depend on the exposed organisms and the methodologies used to conduct experiments (Singer et al 2001; Almeda et al 2014).

There were only a few studies about the toxicity of crude oil on marine crustaceans. *Litopenaeus vannamei* is one of the most commonly farmed crustaceans in the world, in which it potentially spreads throughout coastal and estuary areas releasing from adjacent aquaculture ponds (Senanan et al 2007). Aside from its commercial importance, *L. vannamei* is also an important model organism in ecotoxicology studies not only because of its ecological importance, but also its sensitivity to chemical compounds (Rand 1995), and to both oil water accommodated fraction (WAF) and crude oil exposure in the marine environment (Lee et al 2013). *L. vannamei* that forms its exoskeleton out of calcium carbonate might be susceptible to an increasingly acidic ocean in the future; therefore, the species is also suitable to evaluate the climate change impacts on marine organisms (Orr et al 2005). Thus, the aim of this study was to determine the influence of pH on crude oil WAF toxicity using acute tests to the whiteleg shrimp *L. vannamei.* WAF stock represents water-soluble compounds of crude oil; thus, it has high bio-availability toward test organisms (Singer et al 2001).

Material and Method

***L. vannamei* maintenance**. The individual whiteleg shrimp *L. vannamei* larvae (4 days old) were obtained from a commercial shrimp farm in Lamongan, Indonesia and were maintained in the aquaculture laboratory, Aquaculture Department, University of Brawijaya. These shrimp were cultured in a 160 cm x 50 cm aerated tank with natural seawater until they reached the post-larvae (PL) stage (10-12 days old). The shrimp tank was adjusted and kept to pH 7.5±0.1, salinity 20±0.5‰, and temperature 26±0.5^{o}C. The water was regularly changed to maintain water quality by replacing a quarter of water in the tank with fresh seawater daily. The shrimps were also fed with both formulated feed and brine shrimp.

WAF preparation and PAHs characterization. The crude oil used in this experiment was taken from Wonocolo oilfield. It is a sweet medium-crude oil with 0.15% and 32º Sulphur content and API gravity respectively (Awadh & Al-Mimar 2015). The oil was used to prepare WAF which followed guidelines from CROSERF (Chemical Response to Oil Spills: Ecological Effect Research Forum) with minor modifications. The preparation was conducted at the Laboratory of Reproduction, Faculty of Fisheries and Marine Science, Brawijaya University. WAF was made by diluting 160 mL of crude oil with 1600 mL of sterilized seawater (salinity 20‰) in a sealed 2-L aspirator bottle. The mixture was then mixed in the dark, at room temperature, using a magnetic stirrer with low energy mixing (no vortex, about 200 rpm) to minimize volatilization and degradation of the oil components. After being stirred for 24 hours, the mixture was settled for 1 hour to collect the aqueous phase of crude oil drawn from underneath the whole crude oil at the surface, and used immediately for the acute toxicity experiment (Singer et al 2001).

The total polycyclic aromatic hydrocarbons (PAHs) quantification by gas chromatography (GC) on the initial test solution is required for petroleum toxicity testing (Singer et al 2001). The PAHs of WAF was characterized at the Laboratory of Indonesian Institute of Sciences (LIPI), Jakarta. The Supelco Standard QTM PAH Mix 47930 was used as method of PAH determination using Thermo GC-MS Trace 1310 ISQ LT instrument with Single Quadrupole Mass Spectrometer (320^{o}C) detector.

Preliminary testing. Prior to acute toxicity tests, preliminary testing was performed to determine exposure range concentration and to assure adequate survival control. In brief, twelve triplicate PL *L. vannamei* (10 days old) were exposed for 48 hours to WAF concentration of 0%, 5%, 10%, 20%, 40%, 60%, and 80% in 1-L Erlenmeyer flasks with 500 mL of WAF solution. The flasks were aerated, and the test animals were exposed at pH 7.5, salinity 20‰, temperature 26ºC, and under light/dark regime of 16/8 hours with a ±2500 Lux lamp. Every 12 hours, the water quality (salinity, pH, and temperature) was measured, the test solution was renewed, and the mortality was assessed.

Acute toxicity testing. To evaluate the short-term influence of pH on crude oil WAF toxicity, standard static 72-h acute toxicity tests were conducted using the methods described by Lee et al (2013). Briefly, twelve shrimp were incubated at 26°C in triplicate 1-L Erlenmeyer flasks containing filtered seawater with salinity of 20‰. The shrimps were exposed to WAF concentration of 0%, 25%, 50%, 75%, and 100% with pH concentration of 6.5 and 8.5, in which a total of 30 Erlenmeyer flasks were set up for the experiment.

The pH was adjusted by adding HCL and NaOH to decrease and increase the pH level respectively. The WAF concentration was chosen by considering the result of preliminary testing, while the pH levels are still in the survival range of *L. vannamei* in which no mortality was found on the pH between 5 and 9 (Furtado et al 2015). Thus, it could clearly describe that the mortality of *L. vannamei* was not due to the pH concentrations but the interaction between pH and WAF. The water qualities and the shrimp mortalities were examined at 6, 12, 24, 36, 48, and 72 hours. The mortality data were then used to determine the median lethal concentrations (LC_{50}) using Finney's probit analysis.

Data analysis. The experiments and data analysis were conducted in May-August 2017. The mortalities were calculated according to the formula $M = (M' - C)/(1 - C)$, in which M = rectified mortality, M' = observational mortality, and C = mortality of the control groups (Jiang et al 2012). The LC_{50} based on both WAF and TPH concentrations were calculated by the method of probit units using Minitab 17.1.0. Prior to two-way ANOVA analysis, the data were modeled using normality tests to determine if the data sets were normally distributed. Then, WAF concentrations, pH, and their interaction were used as independent variables of the two-way ANOVA analysis, while the dependent variables were the mortality data.

Results and Discussion

Water qualities of the experimental set up. Salinity, temperature, and pH of both WAF with pH 6.5 and 8.5 were examined at 6, 12, 24, 36, 48, and 72 hours. The water qualities were not significantly changed among WAF concentrations at both pH concentrations (Table 1).

Table 1

The water qualities (mean±standard error) of experimental setup, presented as the average of water qualities 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)	20±0.0	25.75±0.25	6.58±0.21	20±0.4	25.50±0.25	8.44±0.14
25%	20.2±0.4	25.75±0.25	6.56±0.21	20±0.0	25.50±0.00	8.46±0.14
50%	20±0.0	25.25±0.25	6.57±0.20	20±0.4	25.75±0.25	8.48±0.14
75%	20.2±0.4	25.75±0.25	6.51±0.15	20±0.0	25.25±0.25	8.48±0.14
100%	20.2±0.4	25.50±0.25	6.55±0.20	20±0.4	25.00±0.00	8.45±0.14

WAF characterization and its PAHs concentration. The PAHs characterization and quantification using GC-MS revealed fourteen types of PAHs with a total concentration of 8.71 mg L^{-1}. The organic compounds ranged from C_{10} to C_{22} in which benzo[a]pyrene ($C_{20}H_{12}$) had the highest concentration followed by benzo[ghi]perylene ($C_{22}H_{12}$), 2.2 mg L^{-1} and 1.65 mg L^{-1} respectively. Meanwhile, PAH with the fewest number of C atoms (naphthalene) only had a concentration of 0.74 mg L^{-1} (Table 2).

Table 2

WAF characterization and its PAHs concentration

PAHs	*Molecular formula*	*CAS number*	*Concentration (mg L^{-1})*
Naphthalene	$C_{10}H_8$	91-20-3	0.7427
Acenaphthylene	$C_{12}H_8$	208-96-8	0.018
Acenaphthene	$C_{12}H_{10}$	83-32-9	0.2058
Fluorene	$C_{13}H_{10}$	86-73-7	0.471
Anthracene	$C_{14}H_{10}$	120-12-7	0.3117
Phenanthrene	$C_{14}H_{10}$	85-01-8	0.0866
Fluoranthene	$C_{16}H_{10}$	206-44-0	0.1105
Pyrene	$C_{16}H_{10}$	129-00-0	0.6157
Benzo[a]anthracene	$C_{18}H_{12}$	56-55-3	0.5215
Chrysene	$C_{18}H_{12}$	218-01-9	0.4951
Benzo[b]fluoranthene	$C_{20}H_{12}$	205-99-2	0.3874
Benzo[a]pyrene	$C_{20}H_{12}$	50-32-8	2.228
Dibenzo[a,h]anthracene	$C_{22}H_{14}$	53-70-3	0.8658
Benzo[ghi]perylene	$C_{22}H_{12}$	191-24-2	1.6556
Total PAHs			8.7154

Toxic symptoms, mortality and the LC_{50} value of WAF. When the experimental *L. vannamei* were acutely exposed to crude oil WAF for 72 h, the biological activities of individuals decreased, and the body balances were gradually lost, leading to comatose and even death. Some brown adhesive materials and flocs were also observed around their carapaces, mainly in gills. Those manifestations of toxic effects were pronounced in the higher WAF concentrations and longer time exposures.

Furthermore, overall, the WAF toxicity to *L. vannamei* increased over time and exposure concentration in which the higher pH (8.5) had a slightly lower number of mortality than that of lower pH (6.5) ($p < 0.01$). The acute exposure of 75% WAF for 72 h yielded 27.77±12.72% and 38.88±4.81% for pH concentration of 8.5 and 6.5 respectively (Figures 1 and 2).

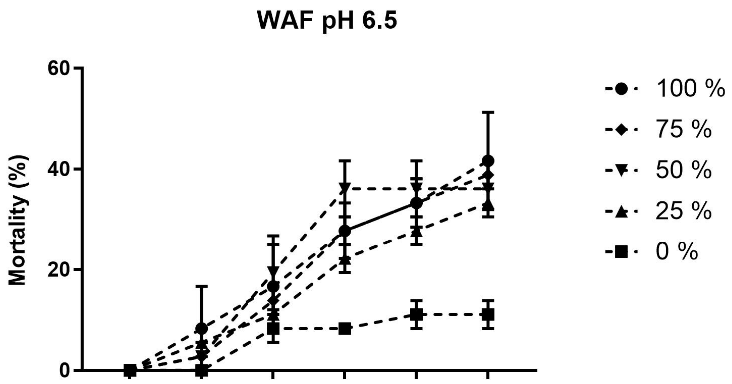

Figure 1. Mortality rate (%) of *L. vannamei* over time and exposure concentration under WAF exposure with pH 6.5.

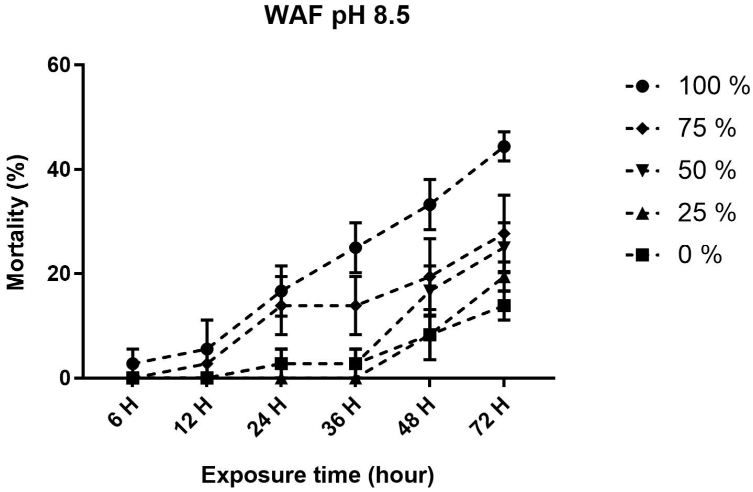

Figure 2. Mortality rate (%) of *L. vannamei* over time and exposure concentration under WAF exposure with pH 8.5.

The LC_{50} values of *L. vannamei* acutely exposed to crude oil-WAF are shown in Figure 3. The tolerances of *L. vannamei* to crude oil pollution stress had significant ($p < 0.05$) differences between pH 6.5 and 8.5 at the same exposure duration in which pH 6.5 had significantly ($p < 0.05$) lower LC_{50} values than that of pH 8.5. For example, the 72-h LC_{50} values of *L. vannamei* were 101.7±9.6 mL L^{-1} and 114.67±11.7 mL L^{-1} for pH 6.5 and 8.5 respectively (Figure 3).

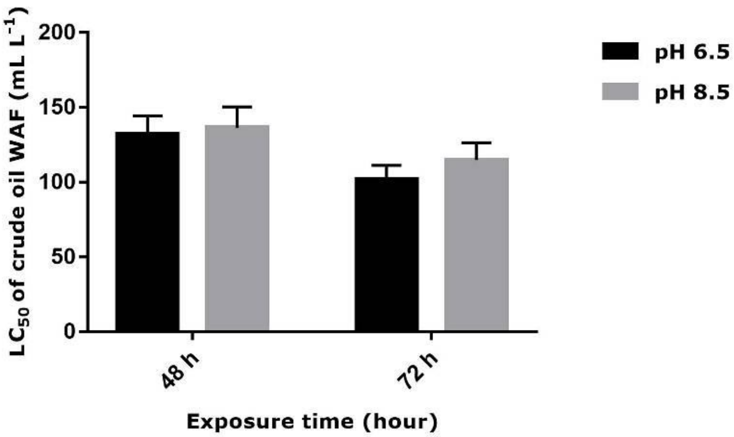

Figure 3. The acute LC_{50} values of *L. vannamei* exposed to crude oil-WAF for 48-h and 72-h.

Discussion. The whiteleg shrimps *L. vannamei* are euryhaline organisms that are able to adapt to a wide range of salinities through ontogeny (Chong-Robles et al 2014). In the aquaculture, the shrimp have optimum growth at a salinity between 20 and 30 ‰ (Amrillah et al 2015). The shrimp live in tropical marine habitats where water temperatures are normally >20°C throughout the year (Kibenge 2016). The shrimp are also tolerant to a wide range of pH between 5 and 9 (Furtado et al 2015). Therefore, the salinity and temperature of the experimental setup were suitable for the growth of *L. vannamei.* Moreover, the pH concentrations of the experiment alone did not significantly impact the mortality, which could interfere with the acute toxicity test results (Furtado et al 2015; Asadi et al 2017b).

Although the mortality rates increased over time and exposure concentrations, the crude oil-WAF is still practically non-toxic to *L. vannamei.* It is based on The United States Environmental Protection Agency aquatic (the US EPA) toxicity classes which categorized toxicant with LC_{50} > 100 µL L^{-1} as practically non-toxic (National Research Council (NRC) 2002). The 72-h LC_{50} values of *L. vannamei* exposed to crude oil-WAF were

> 100% on both pH concentrations (101.7±9.6 mL L^{-1} and 114.6±11.7 mL L^{-1} for pH 6.5 and 8.5 respectively). In another experiment of the toxicity of crude oil-WAF using other crustacean species, the rock pool copepod *Tigriopus japonicus* also showed similar results that the 48-h and 96-h LC_{50} were > 100% (Lee et al 2013). The non-toxic property of crude oil-WAF was also observed on ghost shrimp *Palaemon serenus* that the 96-h LC_{50} were as high as 250 mL L^{-1} (Gulec & Holdway 2000). Meanwhile, the diesel oil-WAF was also non-toxic to marine phytoplankton that the 72-h LC_{50} values for the diatom *Chaetoceros calcitrans* was 46 mL L^{-1} (Asadi et al 2017a).

In order to remedy oil spillage accidents, the dispersant is applied to break up and disperse oil into water columns as tiny droplets for preventing oil coming ashore, which could impact vulnerable coastal ecosystem (Lessard & DeMarco 2000). However, the application enhances the PAHs concentration in the water column, leading to an increase in the toxicity of the oil (Lyons et al 2011). A study on the *L. vannamei* exposed to chemical dispersant showed that the 72-h LC_{50} values were equivalent to 0.19 mL L^{-1} and 0.55 mL L^{-1} for pH 6.5 and 8.5 respectively (Asadi et al 2017b), which is 200-fold more toxic than those of WAF.

PAHs represent some of the most toxic components of crude oil and can bio-concentrate in marine invertebrates (Meador 2003). The non-toxic characteristic of crude oil WAF might be due to the low concentration of PAHs in which the Indonesia's Cepu block crude oil WAF only hold total PAHs of 8.7 mg L^{-1} (Table 2). The crude oil WAF had a high concentration of benzo[a]pyrene (2.2 mg L^{-1}, 25% of total PAHs), which may cause the mortality of the *L. vannamei.* Benzo[a]pyrene is a crystalline, aromatic hydrocarbon consisting of five fused benzene rings that is a potent carcinogen and mutagen (Yu 2002). On the marine polychaete *Perinereis nuntia*, benzo[a]pyrene at concentration of 25 μg L^{-1} caused fertilization success, and hatching decreased by 17% and 46% respectively (Wu et al 2017). However, it needs much higher concentration of benzo[a]pyrene to produce mortality. A study on the toxicity of benzo[a]pyrene on the aquatic crustacean *Artemia salina* showed that the 48-h LC_{50} was 10 mg L^{-1} (Sese et al 2009), which is approximately 4-fold higher than the benzo[a]pyrene concentration presented on the crude oil-WAF used in the experiment.

Another constituent PAHs of the crude oil-WAF was naphthalene which constituted 8% of the total PAHs. A study on the contaminated river of Bangladesh, the Bangsai river, found this carcinogenic compound in almost all of the fish samples in the range of 30-1.000 mg kg^{-1} (Hossain et al 2014); meanwhile, the background concentration of naphthalene in the ocean waters is 1 ng L^{-1} (Neff et al 2011). Therefore, this compound is also bio-concentrated and bio-accumulated as it is absorbed in the fish tissues and accumulates through food chain.

The PAHs of WAF did not contain benzo[a]pyrene and naphthalene alone. In total, there were 14 types of PAHs in the WAF solution which may cause a synergistic toxicity effect on the benzo[a]pyrene and other PAHs. For example, several individual aromatic hydrocarbons containing from 1 to 7 rings have been shown to have synergistic mutagenicity to benzo[a]pyrene (Hermann 1981). Those PAHs may bio-concentrate in the test animal's tissue via surface contacting (Jiang et al 2010), and result in osmotic imbalance due to the respiratory cell disruption (Singer et al 2001). Moreover, the high lipid content of the shrimp which are lipophilic makes it easier for PAHs bounded to the tissues (Chen et al 2014; Carls et al 2016).

Furthermore, the shrimp were slightly more vulnerable and impacted by the crude oil WAF with lower pH. In the study on the influence of pH on the acute toxicity of dispersant towards *L. vannamei* showed that the 72-h LC_{50} on pH 6.5 were 5-fold lower than that of on pH 8.5, meaning that the low pH has synergic toxicity effect on the dispersant (Asadi et al 2017b). However, the 72-h LC_{50} values of crude oil WAF on pH 6.5 were only slightly higher than that of on pH 8.5, which might due to the fact that the WAF itself is practically non-toxic toward the test organisms (Asadi et al 2017a). In calcifying organisms like *L. vannamei* and *Portunus pelagicus,* CO_2-induced pH reduction can change the extracellular acid-base balance disrupting the normal metabolic balance which could impact relevant biological processes such as fitness, metabolism, growth and calcification (Melzner et al 2009; Wu et al 2017).

Conclusions. This study investigated the potential of pH decrease (8.5 to 6.5) to modify the toxicity of crude oil WAF to whiteleg shrimps, *L. vannamei.* The pH of 6.5 slightly but significantly increased the toxicity of crude oil WAF. The 72-h LC_{50} were 101.7±9.6 mL L^{-1} and 114.67±11.7 mL L^{-1} for pH 6.5 and 8.5 respectively. Benzo[a]pyrene may lead the mortality of the shrimps as it represented 25% of the total concentration of PAHs. However, based on US EPA's LC_{50} scale, the crude oil WAF is practically non-toxic to the whiteleg shrimps, *L. vannamei.*

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