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Toxicity of the water-accommodated fraction of diesel on veliger stage larvae of the fluted Giant Clam (*Tridacna squamosa*)

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Abstract. The giant clams (Tridacnidae) found in tropical coral reef ecosystems of the Indo-Pacific are considered endangered, with declining populations throughout most of their range. One anthropogenic impact which could have adverse effects on giant clam populations is hydrocarbon pollution. We conducted an acute toxicity test to assess the effect of the water-accommodated fraction (WAF) of diesel on the mortality, morphology, and behavior of fluted giant clam (*Tridacna squamosa*) veliger phase larvae. The D-veliger larvae (ten per experimental unit) were exposed to different concentrations of diesel (control, 0.1, 1, 2.5, 5, and 10 ml L⁻¹) using a 24-hr static nonrenewal test with four replicates per treatment. The 24-hr LC₅₀ of diesel was 1.14 ml L⁻¹ (CI: 0.65- 1.98 ml L⁻¹) based on Finney's Probit Method. There were no morphological abnormalities (shell deformation) detected at the concentrations tested. However, the expulsion of zooxanthellae was observed at the higher concentrations (2.5, 5, and 10 ml L⁻¹), and the severity of this phenomenon increased in line with diesel concentration. The decreased density of zooxanthellae detected in some living veliger larvae could weaken the larvae and lead to increased mortality.

1. Introduction

Oil pollution has been devastating marine ecosystems ever since the Torrey Canyon supertanker ran aground on a reef off the south-west coast of the United Kingdom in 1967, spilling about 25.36 million gallons of crude oil. The accident marked the first oil spill response and monitoring and effect of oil in the ocean [1]. The most devastating crude oil spill in recent years was the Macondo well explosion from the Deepwater Horizon pipeline platform in April 2010, which released approximately 200 million gallons of crude oil into the Gulf of Mexico, USA [2, 3]. However, oil entering the ocean not only comes from tanker accidents or pipeline blowouts, but an estimated 50% of oil entering the ocean originates from land-based sources such as urban runoff, petroleum consumption by cars, boats, and vessels, and industrial discharges [4, 5].

Petroleum and its refined products can be spilled into the marine environment in various forms, including gasoline; light refined products such as diesel, No. 2 fuel oil, jet fuels and kerosene; crude oil; and heavy distilled products such as No. 6 fuel oil, bunker and heavy survey oils [5]. The toxicity of petroleum and its refined products to marine organisms is mainly due to the water-accommodated fractions (WAF) of the oil, as these enable them to be easily absorbed by aquatic organisms [5, 6]. WAF solutions contain monoaromatic hydrocarbons referred to as BTEX compounds (benzene,

toluene, ethylbenzene, and xylenes), polyaromatic hydrocarbons (PAH), phenols, and heterocyclic compounds, containing nitrogen and sulfur [28]

Light distilled refined products such as diesel (which is mostly used in urban and port activities) are readily dispersed and therefore have great potential to be dissolved in the water column and become bioavailable, thus increasing their toxicity to marine organisms [5, 8-10]. Petroleum products and their WAF can exert a wide range of toxic effects on organisms, from mortality to very subtle biochemical effects. These effects are evident from the early developmental stage through to the adult stage of finfish and invertebrates. Acute exposure to crude oil and petroleum refined products induces mortality in various species, while according to Beiras (2018), the early life stages of marine organisms are more sensitive to exposure than adult forms [4].

Higher mortality occurs in larvae of marine pejerrey (*Odontesthes argentinensis*) when exposed to diesel and gasoline compared to petroleum [10], while 90% mortality and gill damage also occur in milkfish (*Chanos chanos*) juveniles when exposed to the highest concentration (7.5 ml L⁻¹) of diesel oil tested [11]. Mortality rates also increase in egg and larvae of pacific herring and embryos of pink salmon when exposed to more weathered than less weathered crude oil [12-14]. [6] studied six marine organisms exposed to a water-soluble fraction (WSF), oil-in-water dispersion (OWD) and crude oil, finding that the WSF and OWD were more toxic than crude oil to all six marine organisms, which were ranked according to increasing sensitivity to oil as follows: *Cyprinodon variegatus*, *Menidia beryllina*, *Fundulus similis*, *Penaeus aztecus* (post-larvae), *Palaemonetes pugio* and *Mysidopsis almyra*. This study indicated that invertebrate species were more sensitive than fish species when exposed to petroleum products, due to their limited movement, decreasing their ability to avoid exposure [15, 16]. Sublethal effects of morphological and physiological damage occurred in acute and subchronic exposure to the WAF of diesel fuel of the gill pathology of seahorses (*Hippocampus reidi*) [8] and light fuel exposure to mysid shrimp (*Neomysis integer*), leading to increases in oxygen consumption and decreases in ammonia excretion [17]. During the Deepwater Horizon (DWH) Oil spill in 2010, the most affected marine invertebrate was the bivalve *Crassostrea virginica* because the spill occurred at the beginning of their spawning season. Several studies revealed the effects of the DWH oil spill on bivalves were abnormal larval development and reduced survival and subsequent recruitment due to photo-induced toxicity of the WAF [18-22].

The fluted giant clam (*Tridacna squamosa*, Lamarck, 1819) is an important member of coral reef ecosystems and is widespread throughout the Indo-Pacific region. The population has been declining throughout its distribution range, despite the species being listed as under protection [23] and classified by IUCN as lower risk/conservation dependent [24] due to being over-harvested for the meat and shells, habitat loss and anthropogenic impacts such as climate change, pollution and illegal fishing [25]. As a broadcast spawner, the fluted giant clam releases free-floating gametes which develop into free-swimming larvae (veligers) near the water surface, increasing the risk of oil exposure [18]. The success of settlement on the substrate to metamorphose to benthic juveniles depends on the critical stage of transition from veliger to pediveliger [26]. Therefore any disruption such as oil pollution may prevent settlement.

To date, no studies have been done to assess the effect of the water-accommodated fraction of diesel fuel on *Tridacna squamosa* veligers. The aim of this study is, therefore, to evaluate the acute exposure of the larval stage of the fluted giant clam to the WAF of diesel fuels.

2. Materials and methods

2.1. Test organisms

The test organisms used were six-day-old D-veliger stage larvae of *Tridacna squamosa*. The larvae were induced to spawn through a combination of physical and chemical treatment methods [27] from captive parents in the hatchery of Universitas Hasanuddin's Marine Science Station on Barranglompo Island, Makassar, South Sulawesi, Indonesia. Following the spawning, larvae were reared in an aerated 1000 L tank filled with filtered seawater (1µm mesh size), and zooxanthellae were added into the tank on Day 3.

2.2. Test materials

Water-accommodated fractions (WAFs) were prepared from diesel fuel purchased from Pertamina oil depot (filling station). The stock solution of the WAF was prepared according to Loya and Rinkevich (1979), by diluting 10 ml of diesel fuel with 1000 ml filtered seawater (working concentration: 10 ml L⁻¹, designed 100% dilution of WAF) and stirring at 120 rpm for 12 hours using a magnetic stirrer. The solution was left to stand for 4 hours in a separating funnel before the WAF underwent the residual oil was drained in a glass jar and became a stock solution for the acute experiment to allow the separation of the aqueous phase and the residual oil [28].

2.3. Acute exposure

Ten six-day-old D-veliger larvae were randomly distributed and exposed to different concentrations of the water-accommodated fractions (WAFs) of diesel for 24 hours using the acute nonrenewal static test, according to the protocols in APHA (1999) [29]. Static exposures were conducted using four replicates of six concentrations (including control) of diluted diesel WAFs (10; 5; 2.5; 1; 0.1; and 0 ml L⁻¹ or 100%; 50%; 25%; 10%; 5%; and 0% of WAF) in a Petri dish filled with 30 ml of the test solution for each concentration. Water quality parameters, dissolved oxygen, salinity, temperature, and pH were checked at the beginning and end of the static experiment to ensure they had met the criteria recommended by ASTM (2002) [30]. Mortalities and morphological abnormalities were observed and identified at a 6-hour interval and according to Werorilangi et al., (2019) [31], the veliger larvae were considered dead when no movement was observed, the soft tissues were translucent, and the shells were empty due to the expulsion of the mantle tissues and zooxanthellae. Morphological abnormalities were identified using a microscope with 10x10 magnification and photographed at the end of the experiment. All equipment was thoroughly cleaned according to the cleanup procedure in ASTM (2002).

2.4. Data analysis

Mortality data and related concentrations (nominal) were used to calculate median lethal concentration (24h-LC₅₀) and its 95% fiducial concentration interval using Finney Probit Analysis [32]. Differences in cumulative mortality over time of exposure and different concentrations were assessed by Pairwise Comparisons of General Linear Model, and differences in cumulative mortality between treatments and controls were measured with the Dunnett Test. All statistical differences were analyzed using the SPSS application ver 16.

3. Results and discussion

3.1. Mortality

Water quality parameters during the exposure were DO: 5-6 mg L⁻¹, pH: 8-8.4, salinity: 33-34 ppt, and temperature: 26-27°C. There was no mortality in the control treatment, which proved the water quality parameters remained within the limit necessary for the survival of fluted giant clam veliger larvae.

The mortality rate increased over time of exposure and with increasing concentration of diesel WAFs (Figure 1A). The first mortality (2.5%) occurred at the first 12 hours of exposure in the lowest concentration of 0.1 ml L⁻¹, while mortality reached 50% in the highest concentration (10 ml L⁻¹). Only in the two highest concentrations tested (5 and 10 ml L⁻¹) were all larvae dead at the end of the experiment (24 hours). Pairwise comparisons revealed that at 12 hours of exposure, mortalities at concentrations of ≥ 2.5 ml L⁻¹ were significantly higher than in the lowest concentrations (p=0.000), while at 24 hours exposure, mortalities were significantly higher starting at a lower concentration of 1 ml L⁻¹ (p=0.000).

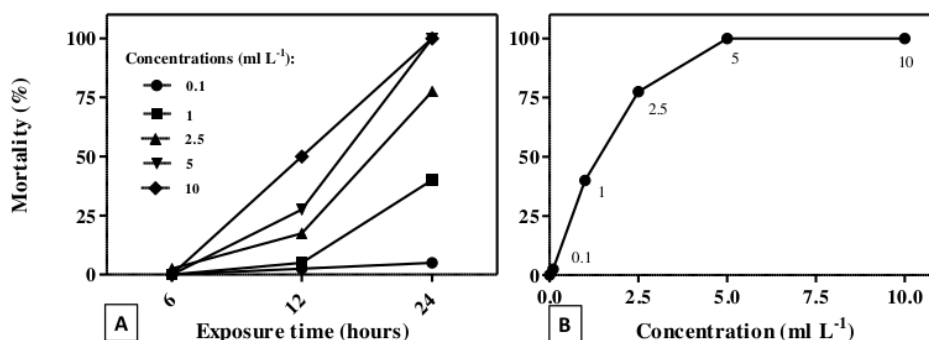


Figure 1. Cumulative mortality over time of exposure (A) and concentration-response relationship for mortality (B) of *Tridacna squamosa* larvae (veliger) exposed to WAF of diesel fuel.

The concentration-response relationship curve showed that cumulative mortality was linearly increasing with increasing concentrations of diesel WAFs (Figure 1B). The Dunnet test showed that the lowest concentration displaying cumulative mortality (77.5%) differed from those in control, starting at 2.5 ml L⁻¹. The median lethal concentration (24-hr LC₅₀) of diesel WAFs obtained from the concentration-response curve (based on a Finney's Probit Analysis) was 1.138 ml L⁻¹ or approximately 11.4% of the WAF dilution (Table 1).

Comparing the results of this study with other studies is challenging because no experimental study on oil toxicity has ever been done on giant clams, while comparing results for *T. squamosa* with those obtained for other marine organisms is also difficult, due to variations in WAF method preparation, different species responses, and life cycles. Table 1 summarizes several studies conducted on the toxicity of the WAF of crude oil and its refined products to marine organisms, mostly focused on early life development. The response was varied from lethal to sublethal effects resulting from oil exposures. Most studies summarized in Table 1 used the bivalves *Crassostrea virginica* and *Crassostrea gigas* from the Deepwater Horizon oil spill (Macondo crude oil). Comparing the 24-hr LC₅₀ of this study with those of *C. gigas* and *C. virginica* [19], our result showed a lower percentage of WAF diesel, causing 50% mortality to the veliger of *T. squamosa*. A study conducted by Saco-Álvarez (2008) [16] using the WAF of Prestige crude oil on *Mytilus galloprovincialis* embryos also showed a higher LC₅₀ than the results of this study, indicating that the WAF of diesel fuel is more toxic to giant clam larvae. A similar range of concentrations to those used in the present study was utilized by Rinkevich and Loya (1977) [33], in which researchers found that 50% of planulae from the coral *Stylopora pistillata* did not survive when exposed to 0.1 ml L⁻¹ of the WAF of Iranian crude oil. Other marine invertebrate larvae, including those from sea urchins and sand dollars, also showed sensitivity to diesel fuel, although on a higher value of LC₅₀ than the results of this study. The toxicity of oil products leading to mortality in the test organisms is likely due to BTEX compounds, which are higher in the WAF of fresh oil than in weathered oil [10, 21]. Most studies also indicate that the early life stages of marine organisms are more sensitive than adult forms, while the negative impact was even greater for the majority of those marine larvae which only had a short-term presence in the impacted areas [20], such as the free-swimming stages of giant clam larvae [18].

Table 1. Summary of several studies on the toxicity response of marine organisms exposed to the WAF of crude oil and its refined products and the median lethal concentration (24-hrLC₅₀) of this study.

Species	Hydrocarbon (WAF)	Response	WAF Chemistry	Concentration Effects	Ref
Bivalvia					
<i>Tridacna squamosa</i> (veliger)	Indonesia (Pertamina) diesel fuel	Mortality	No	24-hr LC ₅₀ : 1.14 ml L ⁻¹ (CI: 0.65-1.98 ml L ⁻¹) or 11.4% of WAF	this study
<i>Crassostrea gigas</i> larvae	Macondo crude oil	Mortality, phototoxic effect	PAH	48-hr LC ₅₀ : >100% of WAF and 19.3 µg L ⁻¹ (tPAH)	[19]
<i>Crassostrea virginica</i> larvae	Macondo crude oil	Mortality, phototoxic effect	PAH	48-hr LC ₅₀ : 31% of WAF and 4.48 µg L ⁻¹ (tPAH)	[19]
<i>Crassostrea virginica</i> (gamete and 1-day old embryo)	Macondo crude oil	Fertilization and abnormal development	PAH	EC ₂₀ : 1650 µg L ⁻¹ (fertilization) and 218 µg L ⁻¹ (abnormal dev.)	[22]
<i>Crassostrea virginica</i> (D-veliger)	Mocondo crude oil	Mortality, fertilization, abnormal development	PAH and TPH	24-hr LC ₅₀ : 1,092 µg L ⁻¹ (WAF); 0.247 mg L ⁻¹ (PAH); 1.348 mg L ⁻¹ (TPH)	[20]
<i>Mytilus galloprovincialis</i> (embryo)	Prestige Oil	Mortality	No	LC ₅₀ : 13% dilution of WAF	[16]
Coral					
<i>Acropora tenuis</i> (larvae)	WAF of condensate of fresh light crude oil	Metamorphosis	TPAH	Metamorphosis inhibition: as low 103 µg L ⁻¹	[15]
<i>Acropora tenuis</i>	Bunker oil, Fuel Oil 467 TM	Mortality	TPH	48-hr LC ₅₀ : 6.1 ppm (3 do <i>A. tenuis</i>), others were less sensitive	[34]
<i>Goniastrea aspera</i>	Bunker oil, Fuel Oil 467 TM	Mortality	TPH	48-hr LC ₅₀ : 6.1 ppm (3 do <i>A. tenuis</i>), others were less sensitive	[34]
<i>Platygyra sinensis</i>	Bunker oil, Fuel Oil 467 TM	Mortality	TPH	48-hr LC ₅₀ : 6.1 ppm (3 do <i>A. tenuis</i>), others were less sensitive	[34]
<i>Stylopora pistillata</i> (planulae)	Iranian crude oil	Survival	No	50% survival starting at 0.1 ml L ⁻¹	[33]
Other invertebrates					
<i>Arbacia punctulata</i> (sea urchin larvae)	Australian fresh diesel fuel	Mortality	No	96-hr LC ₅₀ : >100% of WAF dilution	[9]
<i>Dendraster excentricus</i> (sand dollar larvae)	Australian fresh diesel fuel	Mortality	No	60-hr EC ₅₀ : 27% of WAF dilution	[9]
Fish					
<i>Hippocampus reidi</i> (seahorse)	Diesel fuel	Gill histopathology lesion	No	Increase lesion at 50% WAF exposure	[8]
Chanos chanos (juvenile milkfish)	Diesel fuel	Mortality	No	96-hr LC ₅₀ : 5.12 ml L ⁻¹	[11]
<i>Amphiprion clarkii</i>	Australian fresh diesel fuel	Mortality	No	96-hr LC ₅₀ : >100% of WAF dilution	[9]
<i>Menidia beryllina</i>	Australian fresh diesel fuel	Mortality	No	96-hr LC ₅₀ : 54% of WAF dilution	[9]

3.2. Morphological and behavioral responses

The biological response other than mortality has to be assessed when conducting toxicity testing with marine larvae, because their survival is determined by their ability to move, in order to find food resources and decrease chances of predation [20]. Figure 2 presents photographs of *T. squamosa* D-

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veliger larvae exposed to different concentrations of the WAF of diesel fuel. In the control as well as in all concentrations, the WAF of diesel fuel did not affect larval morphology, 15 all larvae continuing to display a normal, 'straight-hinged D' shape. However, starting at the lowest concentrations of 0.1 and 1 ml L⁻¹, D-veliger larvae displayed stress within portions of the internal granular material (visceral mass), and zooxanthellae were expelled from the shell. The impact became 42 re severe with increasing concentrations of the WAF of diesel fuel starting at 2.5 ml L⁻¹ (where mortality was significantly different from the control). The transparency of the prodissoconch (larval shell) increased in line with increases in concentration, with almost all internal granular material and zooxanthellae being exuded at 5 ml L⁻¹. At the highest concentration of WAF diesel fuel exposure, the mantle cavity of D-veliger larvae was empty, as all internal granular material and zooxanthellae were burst outside of the prodissoconch. In the highest concentrations (5 and 10 ml L⁻¹), all D-veliger larvae were considered dead at 24-hour exposure, 16 en they displayed transparent shells and no evidence of internal organ organization or movement, as described by His et al.,(1999) and Neo et al., (2011) [35,36].

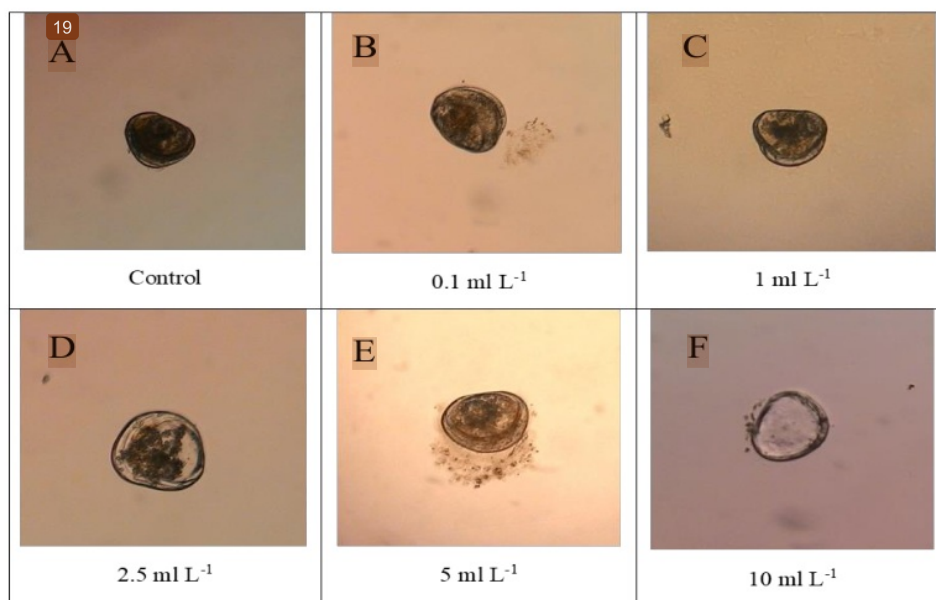


Figure 2. Morphological and behavioral responses of *Tridacna squamosa* D-veliger larvae subjected to diesel exposure. Normal larvae (A); abnormal larvae (B, C, D, E, and F).

Several studies on oil toxicity have revealed that WAF of oil products may cause abnormal development and decreased swimming behavior in free-swimming larvae of marine bivalves [16, 20, 22, 37]. The abnormalities in bivalve larvae 18 e when exposed to a stressor, as described by and His et al., (1997) [38], were the presence of a convex hinge, indented shell margin, incomplete shell, and protruding mantle, alongside the disintegration of tissue or internal granular material [39]. A study was done by Vignier et al., (2015) [22] showed similar results to the present study concerning D-veliger larval abnormalities in *Crassostrea virginica* exposed to Macondo oil 31 24 hours, with results ranging from light to severe moribund and atrophied larvae (Figure 2). The results of visual observations during the current study suggest that despite the absence of morphological shell abnormalities, some larvae appeared to display a stress response starting at the lowest concentrations, represented by the exudation of a portion of the internal granular material. This response was possibly due to acute contraction of the gut, one of the common larval abnormalities leading to mortality, when

marine invertebrate larvae are exposed to oil [40]. The zooxanthellae in the D-veliger larvae of giant clams are located in the gut. Therefore their ejection was also most likely as a result of gut contraction.

4. Conclusions

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The present study has shown the acute toxicity response of D-veliger larvae of fluted giant clam (*Tridacna squamosa*) to the WAF of Pertamina diesel fuel, indicating the sensitivity of fluted giant clam during the free-swimming marine larval stage. Although there was no observed morphological damage to the shell, other behavioral abnormalities were detected, such as the exudation of internal granular material and zooxanthellae, the common symptoms of oil exposure leading to mortalities in the larvae of marine invertebrates. The WAF of Pertamina diesel concentration was found to be significant, with acute toxicity starting at 2.5 ml L⁻¹ (24-hr LC₅₀: 1.138 ml L⁻¹) during a 24-hour exposure period.

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