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Effect of Water Soluble Fractions of Diesel and Biodiesel on Larvae of the Giant Freshwater Prawn, *Macrobrachium rosenbergii*, under Different Thermal Conditions

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Abstract

The present study focused on the comparative effect of the Water Soluble Fraction (WSF) of biodiesel and diesel on larval stages of *Macrobrachium rosenbergii* at different temperatures. The results revealed that biodiesel WSF is less soluble than that of diesel WSF. Both of them have lower water solubility with increasing water salinity. After 96 h, diesel WSF caused approximately 50 % mortality in 8 days old larvae (PL8) at all tested temperatures which were significantly higher than that of biodiesel WSF (less than 15 % mortality) and the control (0 - 10 %) ($p < 0.05$). The mortality (40 % in PL8) decreased when the stage of the larvae increased (less than 20 % in PL13). At 25 °C, the PL13 mortality of both biodiesel and diesel WSF tested larvae was similar and significantly higher than that of the control ($p < 0.05$). However, at 28, 30 and 34 °C the mortality of PL13 became low and was not significantly different with that of the control. For postlarvae (PL22), mortality in all treatments decreased as low as 20 % and was not significantly different among the treatments. The histopathological study shows that biodiesel WSF causes more histological damage to shrimp's tissues than that of the diesel WSF group.

Keywords: Water soluble fractions, diesel, biodiesel, toxicity, thermal conditions, *Macrobrachium rosenbergii*

Introduction

Biodiesel has increasingly gained worldwide attention due to environmental concerns and the limitation of fossil fuels [1]. In Thailand, palm oil has been recognized as a high yielding source of edible oil; it is now used intensively as a raw material for the production of biodiesel, a diesel substitute. Because of its economic importance, demand for palm oil has continuously increased from 2.87 million liter per day in 2013 to 3.21 million liter per day in 2014 [2].

Biodiesel (B100; 100 % biodiesel) in Thailand is produced through the transesterification process of palm oil with alcohol using a sodium hydroxide catalyst. The fatty acid composition of palm biodiesel is palmitic acid (42.6 %), oleic acid (40.5 %), linoleic acid (10.1 %), stearic acid (4.4 %), palmitoleic acid (0.3 %), linolenic acid (0.2 %) and others (1.9 %) [3,4] while petroleum diesel, refined from fossil fuels, is a mixture of hydrocarbons (75 % saturated hydrocarbons, 25 % aromatic hydrocarbons) [5]. Consumers have been encouraged to use various blends of petroleum diesel and palm biodiesel. Two formulas of commercial biodiesel blends are currently available in Thailand; each formula is a mixture of diesel with either 3 % (B3) or 5 % (B5) palm biodiesel. The widespread use of palm biodiesel in Thailand may result in accidental spills into the natural water during either the production process, transportation or storage.

Oil spills in Thailand have frequently occurred in coastal areas that are nursery grounds for aquatic larvae. More than 215 oil spills have been recorded in coastal areas [6]. The water soluble fraction (WSF) of oil has been shown to cause harmful effects to the early stages of aquatic organisms by blocking their ability to swim, inhibiting respiratory activities and/or via direct contact (e.g., eating or skin absorption) [7]. Moreover, gills are the first target organ reacting to pollutants [8]. Two other environmental factors are important to the development of coastal larvae and also alter the impacts of WSF toxicity, temperature and salinity. Temperature affects larval survival [9-12] as well as the toxicity of aromatic hydrocarbons in the WSF. Toxicity is lower at higher temperatures because the aromatics are removed from the water via increased volatilization. Salinity also plays a role in larvae development [13,14] though impacts vary with species and frequently are related to temperature effects [15,16], especially as related to effects of WSF toxicity [17]. Linden *et al*. [17] examined the interactions of temperature (20, 25 and 30 °C) and salinity (10, 20 and 30 ppt) on killifish eggs (*Fundulus heteroclitus*) exposed to No.2 fuel oil at 4 WSF levels (0, 15, 20 and 25 %). They reported the highest mortality at 20 °C and decreased mortality at 25 and 30 °C, as the fuel oil volatilization loss rate was higher at 30 °C than 20 °C and 25 °C; the interaction of salinity and temperature, as related to egg mortality in the presence of fuel oil, were less clear in terms of salinity [17].

Macrobrachium rosenbergii, the giant freshwater prawn, is an economically important native aquatic organism in Southeast Asia that lives in freshwater to brackish water. Their larvae survive only in brackish water [18]. Thailand is the largest giant freshwater prawn producer with about 30,000 metric tons annually [19]. A single study has been conducted with *M. rosenbergii* in regards to WSF toxicity [20]. At 31 and 34 °C, survival of hatched *M. rosengbergii* larvae exposed to diesel WSF was higher than at 25 and 28 °C [20]. Two other *Macrobrachium* species, *M. macrobranchion* and *M. volenhovenii*, are killed by exposure to low concentrations of crude oil WSF possibly because they suffocate from gill membrane blockage [21].

Palm biodiesel has several advantages as a potential fuel source e.g., high oil yield, low sulfur content, low carbon residues, and it is cheaper than other vegetable oils [22]. Production processes and extensive uses of biodiesel could increase the risk of spills into the environment and harmful/adverse effects to living organisms. Leakage of biodiesel into the environment is not officially required to be reported in Thailand and there are no laws in place regarding deleterious environmental impacts related to biodiesel. However, the adverse effects of biodiesel to the environment should be studied as a proper precaution. This study focused on the comparative effect of WSF of biodiesel and diesel on different larvae stages of *M. rosenbergii* at different temperatures. Also, the effect of WSFs on histological alterations in the gills of the tested animals were examined. The results from this research will be useful for determining the risk assessment of biodiesel contamination in the environment.

Materials and methods

WSF preparation

The WSF preparation method was adopted from Anderson *et al*. [23]. Petroleum diesel and biodiesel (B100), were supplied by PTT Public Company Limited and Bangchak Biofuel Co.,Ltd., respectively. Natural freshwater and brackish water (diluted to15 ppt, from concentrated seawater; Samut Songkram Province) were filtered through a 1.2 µm Whatman GF/C before mixing at an oil-water ratio of 1:9. The mixtures were shaken on a horizontal shaker at 180 rpm at 28 °C for 24 h. After settling for 2 h, the water soluble (lower) fractions (100 % WSF) were collected separately and used in further experiments.

Determination of water solubility of diesel and biodiesel

The water solubility of diesel and biodiesel were determined using the partition-gravimetric method 5520B of APHA [24]. Briefly, 200 mL of WFS and 200 mL hexane were added and mixed in a separating funnel. The solutions were allowed to settle until hexane and water were separated before the hexane fractions were removed for analysis. Na_2SO_4 was added to absorb water from the hexane extractions and filtered through Whatman No.1 paper. Hexane was removed by rotary evaporation at 60 °C. Samples were kept in a desiccator at ambient temperature before weighing. The oil concentrations were calculated as follows;

$$\text{oil (mg/L)} = [(A - B)\times 1000]/200 \tag{1}$$

where A is the bottle weight with oil extracted
B is the blank bottle weight

Animal preparation

Healthy *M. rosenbergii* larvae were obtained from the Chonburi Research and Inland Fisheries Development Center, Sri Racha District, Chonburi province. Larvae were maintained in brackish water (15 ppt) 200 L tanks and fed fresh *Artemia* nauplii daily at 0800 and 1600 until used for the experiments in the laboratory of the Center of Excellence for Marine Biotechnology (CEMB), Department of Marine Science, Faculty of Science, Chulalongkorn University. When the prawn larvae reached the age of 8 and 13 days old (PL8 and PL13), they were separated and maintained in brackish water at 15 ppt and the postlarvae (juveniles) age of 22 days (PL22) were maintained in natural filtered freshwater: the WSF toxicity tests were initiated within 6 h of acclimation in the tested environment.

Determination of the effect of diesel and biodiesel WSF

The effects of diesel and biodiesel WSF on *M. rosenbergii* were determined with larvae that were 8, 13, and 22 days old (PL8, PL13, and PL22). The experiments were conducted by rearing individual larvae (n = 18) in a 10 ml single well of a 18 polyethylene culture plate for 96 h. The culture plates were held at 4 temperatures (25, 28, 31 and 34 °C) in temperature-controlled units. The experiments were run in brackish water at 15 ppt for PL8 and PL13 and natural filtered freshwater for PL22 under a 12:12 light:dark photoperiod. The tests on PL8 and PL13 were carried out by diluting diesel or biodiesel 100 % WSF to filtered brackish water 15 ppt at a ratio of 1:1 (50 % WSF) while in PL22 100 %WSF of each oil was used. The tested water was replaced daily with 50 % (vol/vol) of the same experimental water. Each larva was fed daily with 20 *Artemia* nauplii. The mortality of the larva in each well was monitored between 0800 to 0900 every day.

Histological analysis

At the end of the experiment (96 h), PL22 survivors were immediately fixed in Davison's fixative for approximately 24 h and processed using standard histological techniques [25]. The larvae were dehydrated in a series of ethanol, cleared, and embedded in Paraplast. Three larvae in each paraffin block were sectioned at 5 µm thickness and stained with hematoxilin and eosin (H&E). The sections were observed and examined under light microscopy (Olympus BX51) and photographed using a digital camera (Olympus DP71) and the DP Manager program. The morphological abnormalities were derived from fixed images of cells and tissues as seen through the light microscope using digital optical imaging techniques. The presence of histological alterations was evaluated semi-quantitatively based on the severity of the lesions [none (-), 25 % or mild (+), 50 % or moderate (++) and 75 % or severe (+++)]. The alterations were classified in progressive stages of damage to the tissue where stage (-) is an alteration with no change on the normal function, stages (+ and ++) are alterations with severe lesions and impaired normal function of tissue, and stage (+++) is an alteration with very severe and irreversible damage. Ten slides were observed from gill lamellar in each treatment. The method was conducted according to Poleksic *et al*. [26] and Schwaiger *et al*. [27].

Statistical analyses

The concentrations of oil in the WSF fractions of diesel and biodiesel in natural filtered freshwater and brackish waters were analyzed by a t-test. The toxic effects of WSF of biodiesel and diesel with temperature on mortality of different larval stages were determined using descriptive statistics and an analysis of variance (ANOVA) to determine difference of means between treatments.

Results

Water solubility of diesel and biodiesel

The solubility of diesel and biodiesel in freshwater were 1,585±139.17 and 891±210.24 mg/L, respectively while those in brackish water were 630.75±156.24 and 337.81±56.78 mg/L, respectively (**Table 1**). Those values indicated that the solubility of diesel in both freshwater and brackish water were significantly higher than those of biodiesel ($p < 0.05$), and the solubility of diesel and biodiesel in freshwater were significantly higher than those of brackish water ($p < 0.05$).

Table 1 Average concentration of diesel and biodiesel WSF (mean±standard error, n = 3) dissolved in freshwater and brackish water (15 ppt) at 28 °C.

Type of oil	Oil concentration (mg/L)	
	Freshwater	Brackish water
Diesel	$1,585.81 \pm 139.13^{aA}$	864.25 ± 165.49^{aB}
Biodiesel	891.40 ± 210.24^{bA}	337.81 ± 56.78^{bB}

Notes: Means with the same superscript with the small letter are not significantly different in the column and means with the capital letter are not significantly different in the row.

Toxic effect of diesel and biodiesel WSFs on prawn larvae at different temperature

In PL8, WSFs of both diesel and biodiesel caused higher mortalities throughout the experiment and in all temperatures compared to control. The mortality rate of larvae exposed to diesel WSF increased from 0 to almost 50 % during 96 h in all temperatures, while it was always lower than 20 % in the control group. At 25 °C, there was no significant difference between the mortality rate of the larvae exposed to diesel and biodiesel WSFs but they were significantly higher than that of control ($p < 0.05$) while at 28, 31 and 34 °C, the mortality rate of the larvae exposed to diesel WSF was significantly higher than those of biodiesel WSF and the control, respectively ($p < 0.05$) (**Figure 1**).

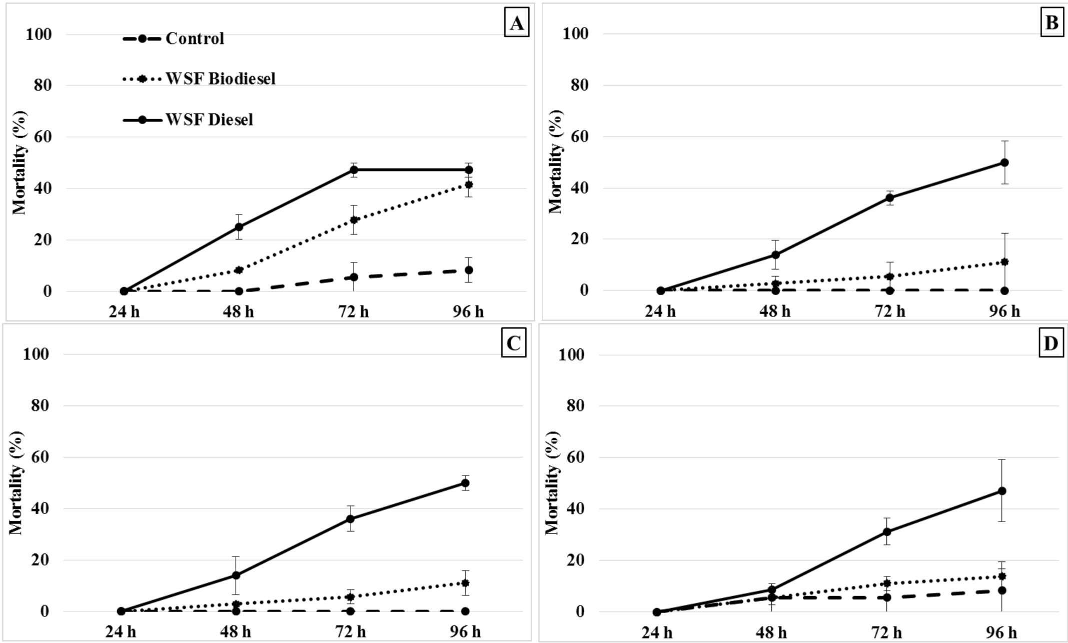

Figure 1 Percent accumulative mortality (mean±standard error, n = 18) exposed to WSF biodiesel and WSF diesel between 24 and 96 h of larvae (LP8) at 25 °C (A), 28 °C (B), 31 °C (C) and 34 °C (D).

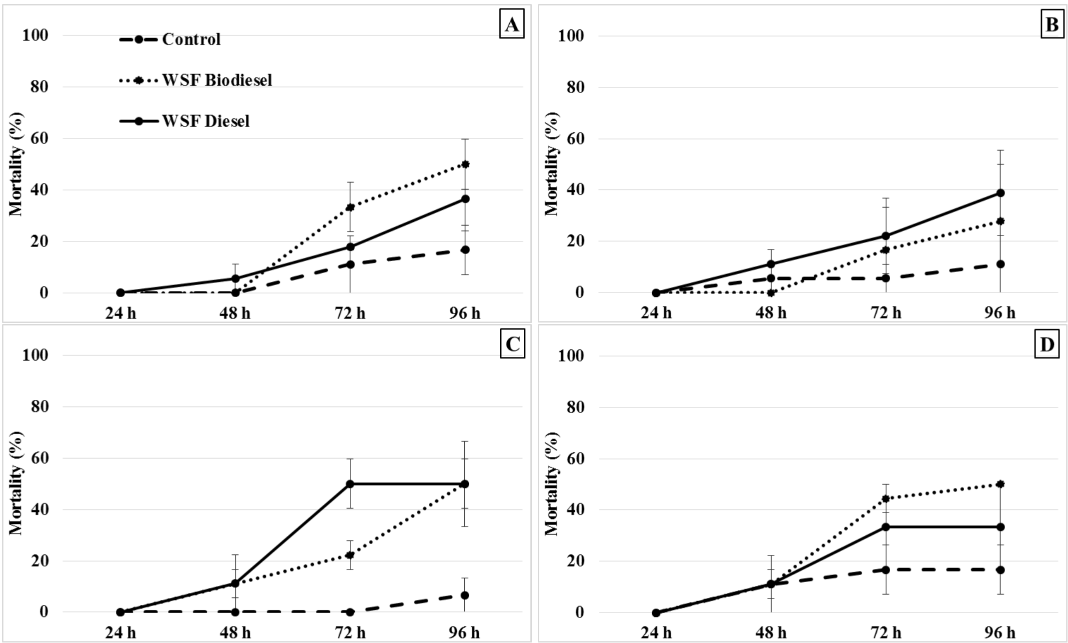

Figure 2 Percent accumulative mortality (mean±standard error, n = 18) exposed to WSF biodiesel and WSF diesel between 24 and 96 h of larvae (LP13) at 25 °C (A), 28 °C (B), 31 °C (C) and 34 °C (D).

In PL13, the mortality rates of larvae in all treatments were increasing with exposing period. There was no significant difference in mortality rates among the treatments and temperature, except at 31 °C where the mortality rate of larvae exposed to diesel and biodiesel WSFs was significantly higher than that of the control ($p < 0.05$) (**Figure 2**).

In PL22 or postlarvae, WSF biodiesel exhibited higher toxicity to larvae than that of WSF diesel and the control. However, less than 20 % of cumulative mortality was detected in larvae treated in all treatments at 96 h and there was no significant difference of mortality among the treatments ($p > 0.05$) (**Figure 3**).

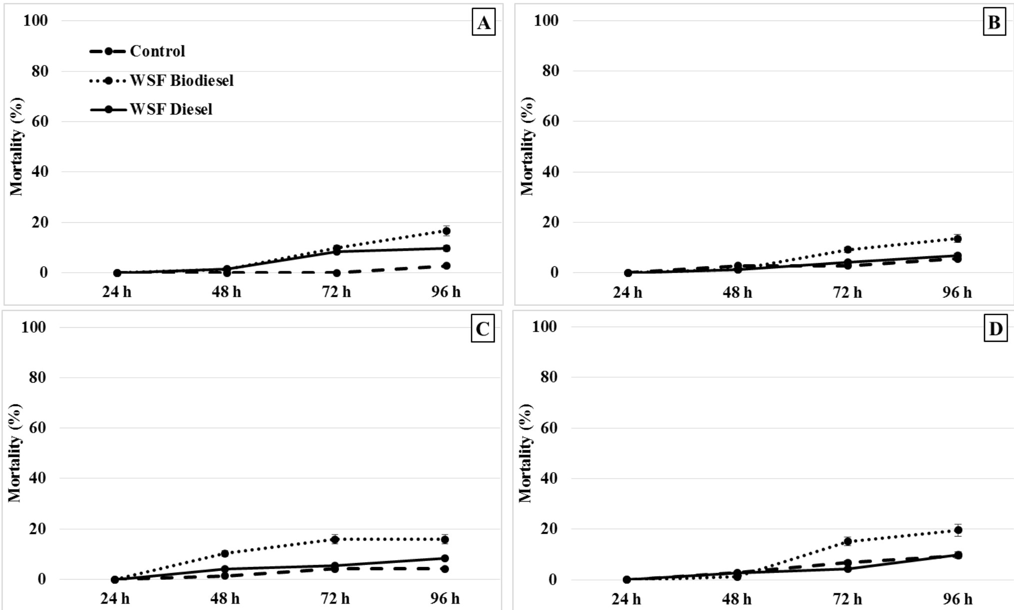

Figure 3 Percent accumulative mortality (mean±standard error, n = 18) exposed to WSF biodiesel and WSF diesel between 24 and 96 h of postlarvae (LP22) at 25 °C (A), 28 °C (B), 31 °C (C) and 34 °C (D).

Histopathological analysis

Histopathological changes of the gill of *M. rosenbergii* larvae exposed to diesel and biodiesel WSFs at 25, 28, 31 and 34 °C after 96 h were examined. The results are shown in **Figures 4 - 6** and **Table 5**. In the control, the histopathological structure of gill lamellar appeared to be normal for larvae maintained at 25 and 28 °C (**Figures 4A** and **4B**). Gill lesions were obviously seen in larvae at 31 and 34 °C (**Figures 4C** and **4D**). These included lamellar disorganization, vacuolar degeneration, swelling of gill lamellar, hemocytic infiltration and eosinophilic cytoplasm.

For larvae exposed to biodiesel WSF, pyknotic of hemocyte and hemocytic infiltration were detected at 25 °C (**Figure 5A**). At 28 °C, gill lamellar disorganization, vacuolar degeneration and swelling of gill lamellar were detected (**Figure 5B**). At 31 and 34 °C, gill lamellar disorganization, atrophy of gill lamellar, vacuolar degeneration, hyaline degeneration, and hemocytic infiltration were clearly detected (**Figures 5C** and **5D**).

For larvae exposed to diesel WSF, vacuolar enlarged and eosinophilic cytoplasm were observed in larve at 25, 28 and 31 °C (**Figures 6A - 6C**) and at 34 °C, severe lesions including gill lamellar disorganization, swelling of gill lamellar and vacuolar enlarged were clearly detected (**Figure 6D**). A semiquantitative scoring of gill lesions shown in **Table 2** is used to compare histopathological effects of different oil WSF (control, biodiesel and diesel) at different temperatures.

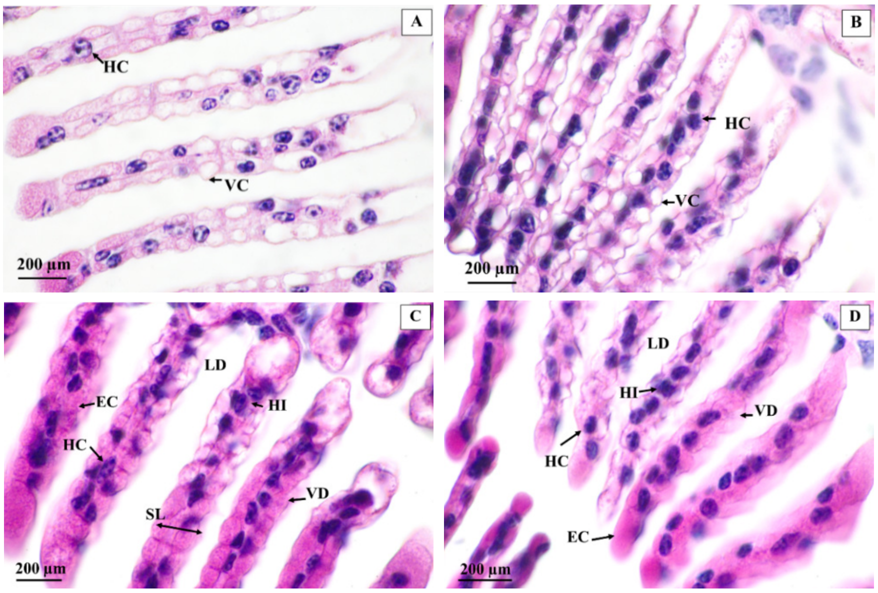

Figure 4 Photomicrograph gill from *M. rosenbergii* postlarvae (PL22) of the control group at 25 °C (A), 28 °C (B), 31 °C (C) and 34 °C (D) at 96 h. A and B were normal gill lamellar consisting of hemocyte (HC) and vacuola (VC). C and D show the effect temperature with lamellar disorganization (LD), swelling of gill lamellar (SL), vacuolar degeneration (VD), hemocyte infiltration (HI) and eosinophilic cytoplasm (EC).

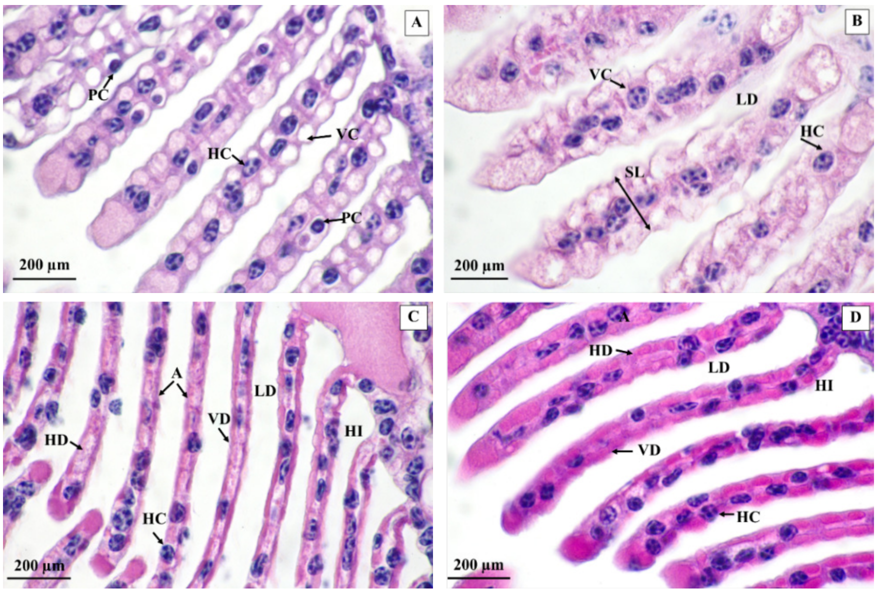

Figure 5 Photomicrograph gill from *M. rosenbergii* postlarvae (PL22) in the biodiesel group at 25 °C (A), 28 °C (B), 31 °C (C) and 34 °C (D) at 96 h. A showed hemocyte (HC), Vacuola (VC), and alternation of gill lamellar; pyknotic of hemocyte (PC). B showed lamellar disorganization (LD) and swelling of gill lamellar (SL). C and D show similar alternation of gill lamellar; atrophy (A), lamellar disorganization (LD), vacuolar degeneration (VD), hyaline degeneration (HD) and hemocyte infiltration (HI).

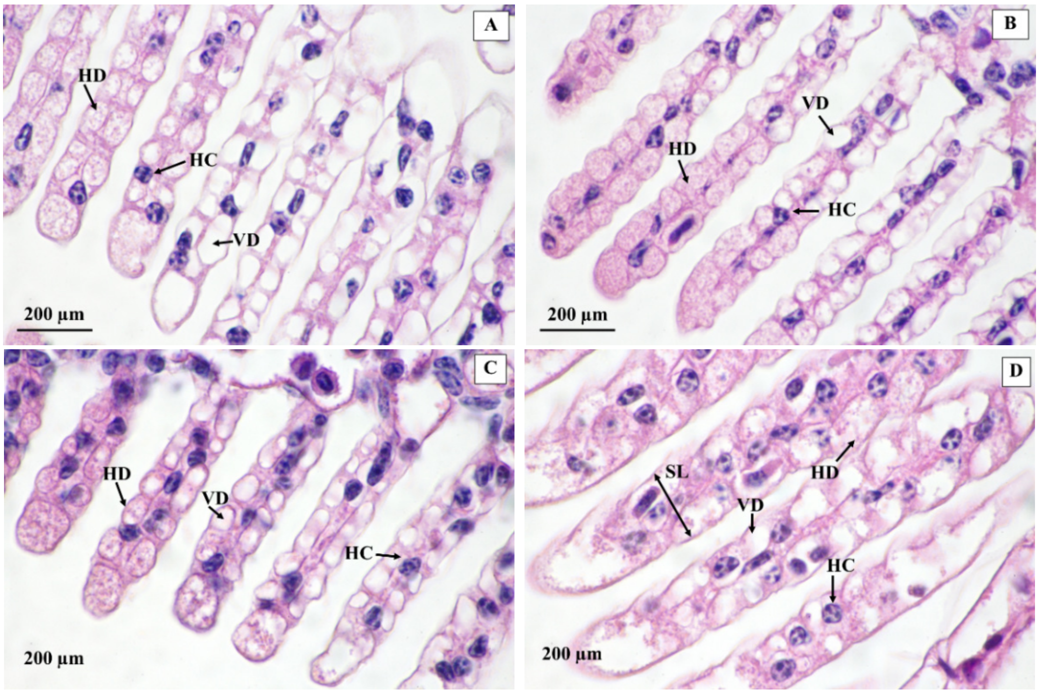

Figure 6 Photomicrograph gill from *M. rosenbergii* postlarvae (PL22) in the diesel group at 25 °C (A), 28 °C (B), 31 °C (C) and 34 °C (D) at 96 h. A-D show hemocyte (HC) and similar alternation of the gill lamellar; vacuolar degeneration (VD), hyaline degeneration (HD), and swelling of the gill lamellar (SL).

Table 2 Gill histopathological alterations of postlarvae *M. rosenbergii* exposure to WSF biodiesel and WSF diesel under different temperatures at 96 h.

Histopathological alterations	Stages of tissue damage	Control				WSF biodiesel				WSF diesel			
		25 °C	28 °C	31 °C	34 °C	25 °C	28 °C	31 °C	34 °C	25 °C	28 °C	31 °C	34 °C
Atrophy	I	-	-	-	-	-	-	+++	+++	-	-	-	-
Gill lamellar disorganization	II	-	-	++	++	-	+++	+++	+++	-	-	-	++
Hemocytic infiltration	I	-	-	++	++	++	-	++	++	-	-	-	-
Hyaline degeneration	II	-	-	++	++	-	-	+++	+++	+	++	++	+
pyknotic nuclei	II	-	-	-	-	++	-	-	-	-	-	-	-
Swelling of gill lamellar	I	-	-	+	-	-	++	-	-	-	-	-	++
Vacuolar degeneration	I	-	-	-	-	-	-	-	-	++	++	++	++

Notes: The score values are none (-), mild (+), moderate (++) and severe (+++) and alternation reversible (stage I) and irreversible (stage II)

Discussion

The present results indicate that WSF of Palm biodiesel is less soluble than that of diesel, and both WSFs become less soluble as salinity increases, supporting previous observations [28-32]. Yassine *et al*. [28] reported solubility of different partitions of diesel and biodiesel depending on aromatic compounds, long chain n-alkanes and FAMEs. Diesel has a significant proportion of aromatic compounds with high electron density that are strengthened by van der Waal interactions with water molecules and allow the ring to form weak hydrogen bonds in water, but biodiesel and n-alkanes do not have this property [28]. The WSF of biodiesel is turbid and formed a thin sheet on the surface, whereas the WSF of diesel is homogeneously dissolved in water [20,28,33]. The different solubility of oil WSFs in freshwater and brackish water found in the present study is possibly due to the salting-out effect [29-31]. The salting-out also affected toxicity of the diesel in brackish water on shrimp larvae. The LC50 (96 h) on *Paemonetes pugio* larvae increased from 1.9 μg/l at 5 ppt to 3.5 μg/l at 15 ppt, respectively, indicating that toxicity of diesel decreases when salinity increases [34].

Our results indicated no difference in the toxic effects of diesel and biodiesel WSFs on PL8 larvae at 25 °C. The biodiesel WSF becomes less toxic to the PL8 larvae when the temperature increases, but not in the diesel group. In PL13, there was no obvious effect of temperature on toxicity of either diesel or biodiesel WSFs. The average mortality of PL13 larvae in all treatments was slightly lower than those of PL8 larvae. It indicated that higher stages of prawn larvae were more tolerant to toxicity of the oils. In postlarvae (PL22), the toxic effect of biodiesel WSF appeared to be significantly higher than that of diesel WSF and showed low mortality rates (less than 20 %). The juvenile prawns are more benthic and can swim up to the water surface to receive more oxygen if it is necessary.

Greater toxicity tolerance in higher larval stages and postlarvae of *M. rosenbergii* on diesel and biodiesel WSF, observed in the present study was also found in *Penaeus* monodon. The toxicities of diesel WSF, fuel oil and lubricating oil on 10 day-old *P. monodon* larvae showed the LC50 (96 h) were 148.97, 34.63 and 7.56 mg/l, respectively, while the 30 day-old larvae were 206.72, 40.71 and 14.14 mg/l, respectively [35]. It can be noted that the toxicity of diesel and biodiesel WSFs shows that the larger sizes of larvae are more tolerable to the oil WSF than the smaller sizes due to the better development of organs and appendages, meaning the larger animals are more effective at getting rid of the contaminants and swimming away from harmful substances [36]. However, the toxicities of diesel and biodiesel WSFs vary vastly depending on species of organisms and sources of biodiesel and diesel feedstock [28,33,36].

Histological abnormal alterations were verified in the gills of tested larvae exposed to diesel and biodiesel WSFs [37]. The lesion severity of the gills in post larvae exposed to diesel and biodiesel WSFs increased as the water temperature increased, especially those from biodiesel WSF and the control group. The increase in temperature enhanced the permeability of the cellular membranes more than 1.4 times through the gill membranes [38], resulting in more degrees of damage to the larvae exposed to higher temperatures.

Gills of postlarvae exposed to diesel and biodiesel WSFs showed a series of alterations which were commonly found in animals exposed to diesel or biodiesel WSFs [37]. Severe damage was observed in the gills of the postlarvae exposed to biodiesel WSF while moderate damage was observed in postlarvae exposed to diesel WSF. It is interesting to note that among the various lesions, gill atrophy is mainly found in larvae exposed to biodiesel WSF, while vacuolar degeneration is only found in larvae exposed to diesel WSF. Atrophy was a cellular adaptation to the harmful environmental changes [39], usually reducing efficiency of oxygen uptake, caused by hydrodynamic changes in the gill chamber [40]. On the other hand, vacuolar degeneration is usually caused by the penetration of dissolved substances which is coincident with the high solubility of diesel WSF [28,41]. Additionally, biodiesel WSF causes a thin film on the water surface that can block oxygen to dissolve into the water but diesel WSF creates a less thin layer on the water surface. The evidence of these abnormal lesions support the results of diesel and biodiesel WSFs toxicities. Thus, atrophy found in exposed larvae can be used as a signature for recognizing the effect of biodiesel WSF, while vacuolar degeneration can be used for indicating the effect of diesel WSF.

In conclusion, evidence obtained from this study reveals that biodiesel WSF is less soluble than diesel WSF and both diesel and biodiesel WSFs are less soluble when water salinity increases. In early larvae stages, diesel WSF is more toxic to *M. rosenbergii* larvae than biodiesel WSF but its toxicity decreases to the same level as biodiesel WSF when the larvae stage increases. Temperature does not alter the toxicity of diesel WSF whereas low temperature (25 °C) appears to increase the toxicity of biodiesel WSFs. Biodiesel WSF becomes less toxic to the larvae at higher temperatures (28 - 34 °C). It appears that biodiesel WSF can cause more histological damage to the tissues of tested larvae than those of diesel WSF, while higher mortality was detected in larvae exposed to diesel WSF. Moreover, the effects of diesel and biodiesel WSFs show some unique histological alterations indicating the different kinds of damage caused by both WSF sources. This difference can be used as a signature for identifying the cause of environmental damages.

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