EnvironmentAsia
The international journal published by the Thai Society of Higher Education Institutes on Environment

Available online at www.tshe.org/EA
EnvironmentAsia 8(1) (2015) 61-67

Effects of Temperature and Water Soluble Fraction of Palm Biodiesel and Diesel Fuel on Hatchability and Survival of First Stage Larvae of *Macrobrachium rosenbergii*

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Abstract

Effects of temperature and water soluble fraction (WSF) of biodiesel and diesel on hatchability and survival of early stage *Macrobrachium rosenbergii* were investigated at the temperature of 25, 28, 31 and 34°C. The purpose of this study was to determine toxic effects of biodiesel and diesel on incubation period, hatchability, and survival of the first larval stage. The results showed a significant difference of incubation period among temperatures. The highest temperature (34°C) resulted in the shortest incubation period (15 days) while the lowest temperature (25°C) gave the longest incubation period (19 days). One hundred percent of hatchability was found at temperature 28 and 31°C in the control group. The lowest hatchability occurred at 100% of WSF of palm biodiesel. The hatchability and survival of eggs through the first stage larvae in control and WSF of biodiesel decreased in higher temperature. However, in 50% WSF of diesel, the highest temperature (34°C) increased the hatchability and survival whereas 100% WSF of diesel, no larval survival could be found. In comparison between WSF of biodiesel and diesel on newly hatched larvae, the diesel was more toxic to the larvae than that of the biodiesel. Regarding temperature and WSF of biodiesel and diesel effects on the first larval stage of *M. rosenbergii*, clearly diesel was more harmful to the larvae than biodiesel.

Keywords: toxicology; temperature; palm biodiesel; diesel; *Macrobrachium rosenbergii*

1. Introduction

In Thailand, biodiesel is widely used as a diesel substitute. Palm biodiesel used as a commercial biodiesel in Thailand are B3, which is composed of 3% biodiesel and 97% diesel, and B5, which is composed of 5% biodiesel and 95% diesel (Department of Alternative Energy Development and Efficiency, 2013). Biodiesel is a production of transesterification process of vegetable oil (lipids) and alcohol with a catalyst (NaOH). The main composition of fatty acids in palm oil are 42.6% palmitic acid, 0.3% palmitoleic acid, 4.4% stearic acid, 40.5% oleic acid, 10.1% linoleic acid, 0.2% linolenic acid, and 1.9% others. (Akoh *et al*., 2007; Robles-Medina *et al*., 2009). Diesel is a fractional distillation of crude oil at 200-350°C. Its compositions is 75% saturated hydrocarbons (parafins including *n*-, *iso*-, and cycloparafins) and 25% aromatic hydrocarbons (napthalenes and alkylbenzenes) (ATSDR, 1995).

Previous records of oil spill exclusively showed in several areas, for example, Kuwaiti oil fires in Kuwait in 1991, Exxon Valdez in Alaska in 1989, BP oil disaster in the Gulf of Mexico in 2010 causing devastation to ecosystem, recreational areas and fishery business. In Thailand, 215 oil spills were reported between 1973-2013 (Department of Marine and Coastal Resources, 2014). Most of them induced mass mortality of aquatic organisms such as fishes and their young planktonic stages which were very sensitive to the environmental changes (Kinne, 1963; 1964; Laughlin and Neff, 1979). Oil pollutants also adversely affected all levels of organisms from biochemical cellular level to population and communities (Capuzzo *et al*., 1987).

Unfortunately, information regarding the effect of biodiesel on aquatic animals have never been reported in Thailand. The objective of the present study was to investigate the effects of biodiesel and diesel fuel on the early stages of *Macrobrachium rosenbergii*, a commercial freshwater prawn in Thailand. It is important to note that hatching areas of freshwater prawn are highly at risk due to various contamination factors, such as oil spill and thermal. The effect of temperature on chemical toxicity was complex. Because temperature alone may be lethal, and the toxicant may alter lethal thermal limits (Cairns *et al*., 1975). This study focuses on the effect of WSF of palm biodiesel and diesel on egg hatchability and survival of first larval stage of *M. rosenbergii*.

2. Materials and Methods

2.1. Experimental design

A completely randomized block design was used in this study. Two types of fuel oils; palm biodiesel and diesel were used to find their toxicity to egg and early larval stages of *M. rosenbergii* at the temperatures of 25, 28, 31 and 34°C.

2.2. Preparation of oil water soluble fraction

Oil toxicity test was prepared as water soluble fraction (WSF), a common practice for oil toxicity test of aquatic organism (GESAMP, 1993). Dechlorinated water was prepared for egg hatchability experiment and 15 psu brackish water was used for the first larval stage experiment. All water was filtered through a Whatman GF/C 1.2 µm paper before used. WSF was prepared using one part of oil mixed with nine parts of designed water, shaken by an incubator horizontal shaker 180 rpm at temperature 25, 28, 31 or 34°C for 24 h (Anderson *et al*., 1974). After settling for 2 h, the lower part was separated as 100% of WSF for future experiment. A partition gravimethod 5520B (APHA, 1995) was used to determine total oil content in WSF.

2.3. Shrimp and egg preparation

Male and female prawns were selected from a local farm, disinfected in cleaned seawater for a few min, reared in 250 L freshwater aquarium with sub-sand filter and fed twice daily at 8.00 am and 16.00 pm with commercial prawn diet.

2.3.1. Preparation of eggs for effect of oils on hatchability

On the first day, fertilized gravid female was separated and the early stage of eggs were gently isolated from the brood chamber with a small brushes in filtered seawater and the healthy eggs were selected under a compound microscope for experiment.

2.3.2. Preparation of the first stage larvae

Berried female with grey eggs was isolated and reared in aquarium containing 15 psu water. After hatching, the healthy hatched larvae were selected and fed with *Artemia* sp. 3 times daily.

2.4. Effect of biodiesel and diesel on egg hatchability

Effect of temperature and WSF of biodiesel and diesel on egg hatchability was determined in 24 well-culture plates at the temperature of 25, 28, 31 and 34°C with different concentrations of WSF. Five milliliters of tested WSF was filled in each well and 5 early stage eggs were incubated in the wells. Each day 50% of the same fresh prepared WSF was exchanged. Egg development and survival were checked daily with magnification glass. The experiment was ended when all eggs were hatched. Dead eggs were removed immediately. All treatments were maintained in 12 h light/12 h dark at controlled temperature.

2.5. Effect of biodiesel and diesel on larva I

Effect of temperature and WSF of biodiesel and diesel on survival of larva I was determined in 18 well-culture plates, at the temperature of 25, 28, 31 and 34°C in different concentrations of WSF. Ten milliliters of WSF was filled in each well with only one newly hatched larva. Each day 50% of the same freshly prepared WSF was exchanged. Larval development and survival were checked with magnification glass daily. The experiment was ended at 96 h. All treatments were maintained in 12 h light/ 12 h dark at the controlled temperature.

2.6. Statistical analyses

Effect of temperature and WSF of oils on hatchability, incubation period and hatching survival were tested by ANOVA. A probit analysis was used to find LC_{50}-96 h of WSF's on prawn larva I.

3. Results and discussion

3.1. Water soluble fraction (WSF)

Average total lipid content in WSF of biodiesel and diesel at different temperatures was shown in Table 1. Diesel and biodiesel solubility were significantly different ($p<0.05$) in all temperatures. Biodiesel significantly increased solubility with higher temperature. At a temperature of 25°C, oil content in WSF of biodiesel was 2,180.13 mg/L whereas at 34°C the oil content in WSF increased to 4,181.81 mg/L. Solubility of diesel was significantly high at 34°C (5,136.2 mg/L). By observation, the WSF of biodiesel was likely milky solution with a fine sheet covered on the surface while the diesel was well dissolved in water with glossy on the surface as reported by Birchall *et al*. (1995) and Demello *et al*. (2007).

3.2. Effect of temperature and WFS of biodiesel and diesel on egg hatchability

Effects of temperature and WSF of biodiesel and diesel on egg development (incubation period) are

Table 1. Average oil content in WSF of biodiesel and diesel (mean+SD) at different temperature.

Temperature (^{o}C)	Solubility of oil (mg/L)	
	Biodiesel	Diesel
25	2,180.13±871^{cB}	4,597.88±681^{bcA}
28	3,601.32±475^{abB}	4,238.67±728^{bcA}
31	3,549.68±924^{b}	3,941.94±1147^{c}
34	4,181.81±1167^{aB}	5,136.24±842^{aA}

Note: Means with different small letter of superscript are significant differences in column and with different capital letter superscript are significant differences in row (*p<0.05*).

shown in Table 2. The results revealed that temperature in this study were significantly affected prawn's egg development. Incubation period was 19, 17.4, 16.8 and 14.9 days at temperature 25, 28, 31 and 34^{o}C, respectively. The same result was also recorded by Ogasawara (1984) and Manush *et al*. (2006). There was no WSF's effect on egg incubation period (*p>0.05*).

Hatchability of prawn's eggs was almost complete (100%) in the control group without any temperature effect (Table 3). Hatchability was slightly lower in oil WSF treatments at temperature between 25 and 31^{o}C. But, treatments with 100% WSF of both biodiesel and diesel showed significant reduction of egg hatchability at 34^{o}C. This might be metabolic effects due to high temperature factor with a co-effect of WSF of the oils. Egg hatchability in 100% WSF of biodiesel was lower than that of 100% WSF of diesel in all temperatures. It was clear that 100% WSF of biodiesel formed emulsion with water, easily clotted the respiratory pores of egg surface, reduced oxygen supply of the embryo, and caused failure to larval hatching. Our results also suggested that optimum temperatures for egg hatchability in all treatments were between 28 and 31^{o}C. These were in accordance with optimal embryonic development temperature for *M. rosenbergii* (Sebastian, 1996).

Normally, larvae could swim freely in the water column after hatching. However, in water with WSF of oils, the larvae might die immediately after hatching. Table 4 indicated the percent survived hatching larvae at different WSF of oils and temperature. In control group, hatched larvae survival was 86-98% with the lowest rate at the highest temperature (34^{o}C). WSF of biodiesel at 50% and 100% was effected on hatching and survival of larvae. The higher WSF of biodiesel caused the higher mortality of hatching larvae. Biodiesel was found to be more toxic to early hatching larvae at temperature 34^{o}C. In contrast, diesel seemed to be dramatically toxic to hatching larvae. At 100% WSF of diesel, no larva could survive after hatching. At 50% WSF, larvae at lower temperature (25 and 28^{o}C) also died at hatching, but more larvae significantly survived at higher temperature (31 and 34^{o}C). The results clearly showed that 100% WSF of diesel was extremely toxic to prawn's larvae and caused mortality to larvae in all temperatures. Biodiesel and diesel presented

Table 2. Effect of temperature and water soluble fraction of biodiesel and diesel oil on egg incubation period (spawning to hatching). Values are mean ± SD, n=24.

Temperature ($^{\circ}$C)	Incubation period (days)				
	Control	50% WSF biodiesel	100% WSF biodiesel	50% WSF diesel	100% WSF diesel
25	19.00±0.00^{a}	19.00±0.00^{a}	19.00±0.00^{a}	18.96±0.20^{a}	19.00±0.00^{a}
28	17.21±0.78^{b}	17.33±0.48^{b}	17.00±0.00^{b}	17.62±0.49^{b}	17.63±0.49^{b}
31	16.96±0.20^{c}	16.92±0.28^{c}	16.37±0.49^{c}	16.92±0.28^{c}	16.58±0.50^{c}
34	15.00±0.00^{d}	15.00±0.00^{d}	14.54±2.25^{c}	15.00±0.00^{d}	14.96±0.20^{d}

Note: Means with different small letter of superscript are significant differences in column and with different capital letter superscript are significant differences in row (*p<0.05*).

Table 3. Effect of temperature and water soluble fraction of biodiesel and diesel oil on prawn's egg hatchability. Values are mean ± SD.

Temperature ($^{\circ}$C)	% hatchability				
	No oil	50% WSF biodiesel	100% WSF biodiesel	50% WSF diesel	100% WSF diesel
25	99.17±1.67^{A}	97.55±3.10^{A}	88.13±1.97^{aB}	100.00±0.00^{abA}	95.89±4.21^{abA}
28	100.00±0.00^{A}	94.90±4.53^{AB}	89.14±8.73^{aB}	98.33±1.92^{abA}	94.60±4.55^{abAB}
31	100.00±0.00	96.55±6.90	99.19±1.61^{a}	96.55±2.82^{ab}	99.19±1.61^{a}
34	100.00±0.00^{A}	97.47±1.69^{A}	64.66±14.95^{bB}	90.15±7.81^{bA}	89.09±5.98^{bA}

Note: Means with different small letter of superscript are significant differences in column and with different capital letter superscript are significant differences in row (*p<0.05*).

Table 4. Effects of temperature and water soluble fraction of biodiesel and diesel oil on the percentages of survived larvae after hatching. Values are mean±SD.

Temperature (°C)	Hatchability and survival (%)				
	No oil	50% WSF biodiesel	100% WSF biodiesel	50% WSF diesel	100% WSF diesel
25	97.50±1.67^{aAB}	96.75±2.63^{aA}	83.91±1.56^{aB}	0.83±1.67^{bC}	0.00±0.00^{C}
28	98.39±3.23^{aA}	93.11±7.75^{aAB}	86.58±7.15^{aB}	0.00±0.00^{bC}	0.00±0.00^{C}
31	96.75±4.56^{aA}	93.32±8.00^{aA}	93.38±8.00^{aA}	10.65±11.58^{bB}	0.00±0.00^{C}
34	86.72±7.76^{bA}	73.05±6.52^{bC}	33.36±7.92^{bC}	37.39±6.35^{aC}	0.00±0.00^{D}

Note: Means with different small letter of superscript are significant differences in column and with different capital letter superscript are significant differences in row ($p<0.05$).

controversy effects to all temperatures. Biodiesel seemed to be more toxic to newly hatching larvae at higher temperature while less toxicity was found in diesel group at temperature above 31°C (Table 4). Diesel is more aromatic hydrocarbon than biodiesel and may be affected by high temperature which showed the greatest loss rate at highest temperature (30ºC) (Laughlin *et al*., 1979). Additionally, aromatic hydrocarbons retained toxicity in cold water longer than in warm water (Rice *et al*., 1976; 1977).

3.3. Toxicity of WSF of oils and temperature on the first larval stage

Effects of biodiesel (Fig. 1) and diesel (Fig. 2) on mortality of the first larvae of *M. rosenbergii* were significantly different in all temperature treatments. Biodiesel was less toxic to the larvae, even at 100% WSF. The larval mortality was only 20% at 96 h exposure and there was no significant difference to the control group (Fig. 1). Toxicity of WSF of biodiesel on zooplanktons and larvae of *Daphnia mangna, Menidia beryllina*, and *Mysidopsis babia* was reported that emulsions of tiny droplets of biodiesel coating on appendage and gill affected their mortality (Wedel, 1999; Hollebone *et al*., 2008; Khan *et al*., 2007). Biodiesel was a physical harmful effect on motivation and respiration of zooplankton and larvae. It was noted that temperature (25 to 34°C) had no effect on larval mortality in the biodiesel experiment over 96 h exposure.

Fig. 2 showed percent accumulated mortality at WSF of diesel at the temperature of 25 to 34°C. Diesel revealed a higher toxicity to *M. rosenbergii* larvae (Fig. 2 A-D) than that of biodiesel (Fig. 1 A-D). High % WSF of diesel increased mortality of

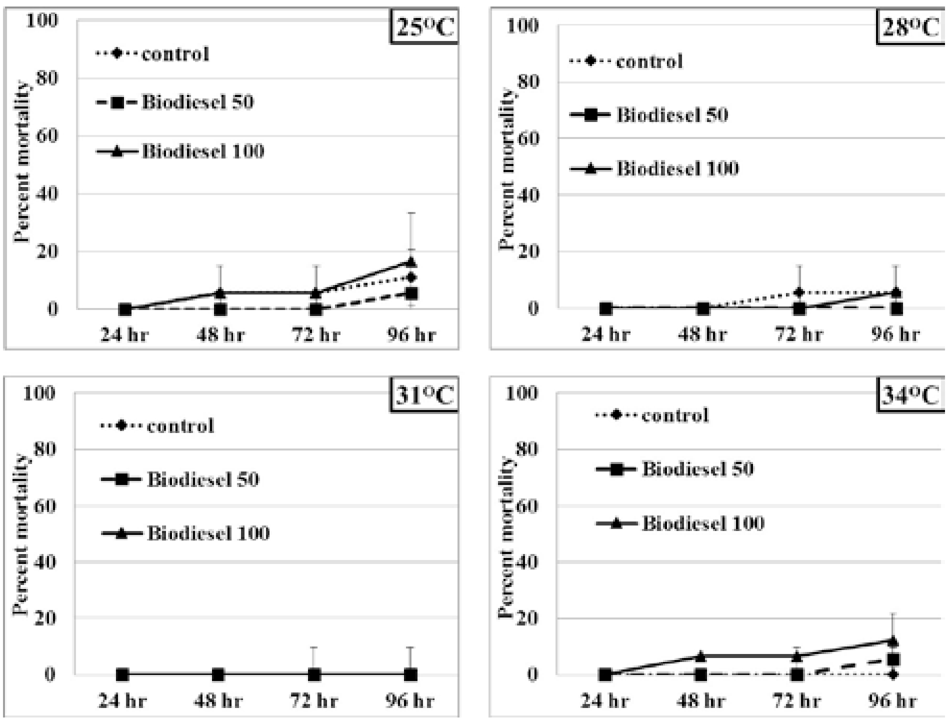

Figure 1. Percent accumulated mortality (mean±SD) at 24-96 h of the stage I larvae at different %WSF of biodiesel and temperature.

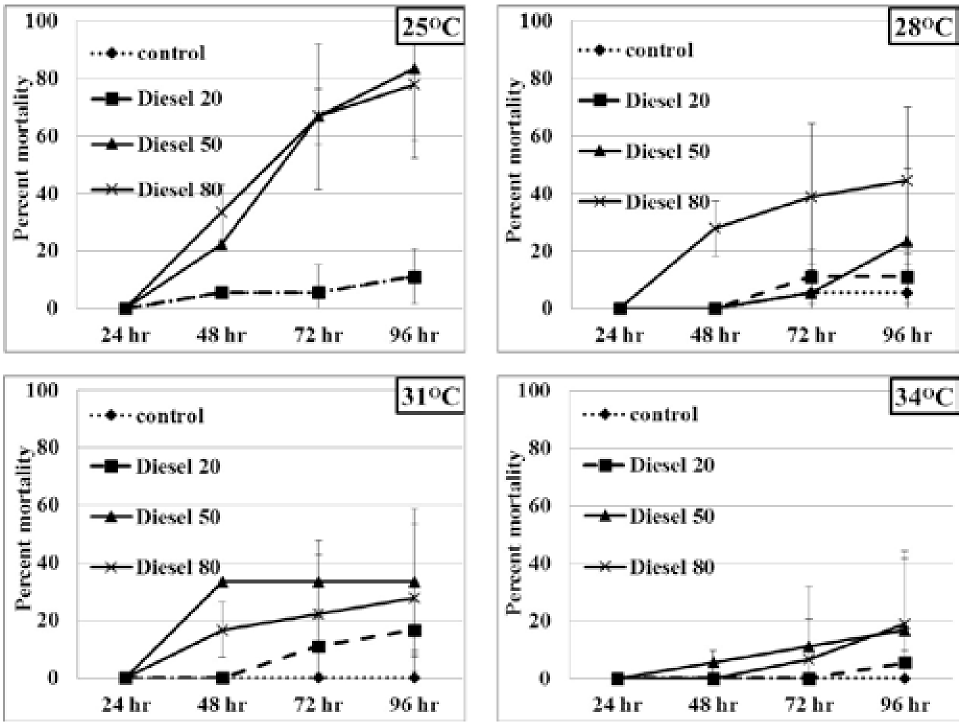

Figure 2. Percent accumulated mortality (mean±SD) at 24-96 h of the stage I larvae at different %WSF of diesel and temperature.

the first stage larvae. In addition, temperature also affected percent mortality at different WSF's of diesel. Clearly, diesel's toxicity decreased with an increase of temperature. At temperature of 34°C, the percent mortality was the lowest. Using probit analysis, we found that median lethal concentration at 96 h (LC_{50}-96 h) of diesel decreased markedly with increasing temperature (Table 5). The effect of temperature on diesel toxicity of the first stage larvae might be explained by the same reason with larval hatching and survival.

The results of temperature and WSF of diesel showed lower mortality of prawn first larvae at higher temperature. This might be due to volatility of diesel's WSF at high temperature (Benjumea *et al*., 2008). A report on acute toxicity of crude oil on marine copepods also indicated the decreased toxicity of oil with increasing natural water temperature (Jiang *et al*., 2012).

Survival of prawn larvae in biodiesel was temperature independent, but in diesel was temperature dependent. At higher temperature less toxicity of diesel

Table 5. Water soluble fraction of diesel at LC_{50}-96 h of the first stage larvae of *M. rosenbergii* in different temperature.

Temperature (°C)	LC_{50}-96 h (% WSF)
25	1.334
28	5.023
31	15.157
34	36.05

was found. WSF of biodiesel was less toxic than that of diesel on prawn larvae. Birchall *et al*., (1995) reported that marine diesel toxicity on *Daphnia magna, Gammarus pulex* and *Lymnaea peregra* were higher than that of rapeseed biodiesel. Wedel (1999) also reported a less toxicity of WSF of soy methyl ester biodiesel than that of WSF of diesel on shrimp larvae (*Mysidopsis bahia*) and fish larvae (*Menidia beryllina*).

Biodiesel dissolved in water as WSF and set calmly for a few hours. Tiny droplets were formed and possibly harmed or interfered to appendages and gills of the larvae. The larval mortality may be caused by physical damage, not the chemical toxicity. Hollebone *et al*., (2008) found that toxicity of WSF of biodiesel (soy, canola and animal) was lower than that of low sulfur and ultra low sulfur diesel about five to 10 times. They reported that mortality of tested animals was caused by physical smothering (trapped with oil droplets). Khan *et al*., (2007) showed that LC_{50}-24 h of *D. magna* was 1.78 mg/L for petrodiesel and 4.65 mg/L for biodiesel while LC_{50}-96 h on rainbow trout was 578.13 mg/L for petrodiesel and 1073.54 mg/L for biodiesel. This indicated that toxicity of WSF of oil not just depended on oil types, but it also depended on species and forms of living. However, the planktonic forms of crustaceans are usually more sensitive than the adult forms (Katz, 1973; Neff *et al*., 1976; Laughlin *et al*., 1978; Laughlin and Neff, 1979). Lavarías *et al*. (2004) reported that postlarvae of *M. borellii* were more sensitive than adults exposing to WSF of crude.

4. Conclusion

The effects of WSF of biodiesel and diesel, temperature on egg development, hatchability, survival, and mortality of the first stage larvae of *M. rosenbergii* were investigated. It can be concluded that egg developed faster at higher temperature without any oil WSF's effect. For hatchability, only 100% WSF of biodiesel at a temperature of 34°C significantly reduced egg's hatching rate compared to other WSF and temperature effects. The first hatched larvae mortality was affected by high temperature. At 34°C, more mortality took place both in the control and in WSF of biodiesel treatments, however, in 50% WSF of diesel, less mortality was observed. No hatched larvae survived in a treatment of 100% WSF of diesel at 34°C. As a result, for temperature and WSF of biodiesel and diesel effects on the first larval stage of *M. rosenbergii*, diesel was found to be more harmful to larvae than that of biodiesel. However, toxicity of diesel decreased with increasing temperature due to its instability.

Acknowledgements

This study was supported by the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund). We gratefully acknowledge to PTT Thailand, Pure Biodiesel Co., Ltd, and Bangchak Biofuel Co., Ltd. Thanks for the Center of Excellence for Marine Biotechnology (CEMB), Department of Marine Science, Faculty of Science, Chulalongkorn University for all the support. Thanks also to Dr. Narongsak Puanglarp, Center of Excellence for Marine Biotechnology (CEMB), Chulalongkorn University for his assistance in English edition.

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Received 13 August 2014
Accepted 22 September 2014

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