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RESEARCH PAPER

The Toxicity of 10W-40 Motor Oil on Zebrafish Early Life Stages

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Abstract

In this study, toxicity of water associated fraction of 10W-40 motor oil was investigated during the early life stages of zebrafish (*Danio rerio*). In 1.5-hour post fertilization (hpf), the fertilized zebrafish eggs were exposed to the oil fraction (1.25, 2.5, 5.0,10.0, 20.0 and 40.0 v/v) and were followed through 24, 48, and 72 hpf. The morphological endpoints of development toxicity of the oil in zebrafish embryos were observed under a stereomicroscope. Higher concentrations caused acute lethal effects, and lower concentrations caused sublethal effects, such as incomplete gastrulation, tail curvature, weak pigmentation, head malformation, pericardial edema, vertebra column defect and scoliosis. Moreover, with the increase of the oil concentration, the malformation rate increased. The hatching rate and body length were also decreased. Finally, it was found that the water associated fraction of 10W-40 motor oil affected the normal embryonic development of zebrafish.

Keywords: Semisynthetic car oil, *Danio rerio*, embryonic development, toxicity.

10W-40 Motor Yağının Zebra Balığı Embriyolarının Erken Gelişim Evreleri Üzerine Toksisitesi

Özet

Bu çalışmada 10W-40 motor yağının gelişimsel toksisitesi zebra balığı (*Danio rerio*) embriyolarında araştırılmıştır. Fertilizasyondan 1.5 saat sonra döllenmiş balık yumurtaları motor yağının sulandırılmış haline maruz bırakılmış (1.25, 2.5, 5.0,10.0, 20.0 and 40.0 v/v) ve maruziyet 72 saat sürdürülmüştür. Motor yağının zebra balığı embriyolarında sebep olduğu gelişimsel toksisitenin morfolojik belirtileri stereomikroskop altında takip edilmiştir. Motor yağının yüksek konsantrasyonları akut ölümlere yol açmış, düşük konsantrasyonlarda ise embriyo ve larvalarda tamamlanmayan gastrulasyon, kuyruk kıvrılması, zayıf renklenme, baş anormalliği, perikardiyal ödem, omurga sütun defekti ve skolyoz gibi subletal etkiler gözlenmiştir. Üstelik motor yağı konsantrasyonunun artışıyla beraber malformasyonda da bir artışın olduğu belirlenmiştir. Aynı şekilde koryondan çıkışlar gecikmiş ve larva boyu da kısa kalmıştır. Sonuç olarak yapılan çalışmada 10W-40 motor yağının sulu fazının zebra balığı embriyolarının normal gelişimini olumsuz etkilediği bulunmuştur.

Anahtar Kelimeler: Yarı sentetik motor yağı, *Danio rerio*, embriyonik gelişim, toksisite.

Introduction

Pollutants may enter aquatic systems as the result of human activity in various ways such as unintended or intended release, discharge of sewage or industrial effluents etc. A further problem is the release of oil from tankers, most dramatically in the case of shipwrecks, when large quantities are discharged in a relatively short time in one area. It is estimated that 3.2 million tons petroleum hydrocarbons log into to the marine environment every year (Walker *et al.*, 2004). Spillage of crude oils is major source of hydrocarbon pollution.

Engine or motor oils are obtained from refining the crude oil. Crude oils are characteristically 75 to 85 percent base stock which is combined with additives. The base stock is formed a synthetic and mineral oil. Synthetic motor oils have been manufactured by polymerizing short chain hydrocarbon molecules. The molecules are named alpha-olefins (AOs) and polyalpha-olefins (PAOs). Synthetic motor oils seem chemically similar to mineral oils purified from crude oil. But, PAOs don't include the dirtiness or waxes inherent in conventional mineral oils. PAOs compose widely used synthetic engine oil in Europe and United States (Randles *et al.*, 2007). Used and unused motor oils that are infiltrated, dumped or rejected may join stormwater. Nevertheless, the extent to used motor oil

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are contaminating stormwater, and the final receiving waters is unknown (OEHHA, 2006). Because of motor oil's chemical composition, worldwide dispersion and potentially adverse effects, it can be an important environmental problem (ATSDR, 1997).

Crude oils are naturally occurring, exceedingly complex mixtures, which consist predominantly of hydrocarbons, and contain sulfur, nitrogen, oxygen and metals as minor constituents. Motor oils and lubricants are among the products derivable from crude oil. Each type of crude oil or refined product has distinct physical properties which affect the way the oil spreads and breaks down, and ultimately determines the hazard it may pose to marine and human life (Aluyor and Ori-jesu, 2009). Due to the loss of motor oil to the wetland and water systems by motor punctures, spills, and illicit elimination, definition of adverse effects of motor oils are of ecological value. Used and unused motor oil pollution is now converting a serious problem, and therefore requires attention. Unfortunately, the data are scarce on motor oil toxicity (Ramadas *et al.*, 2015).

Zebrafish (*Danio rerio*) have been extensively used for toxicological studies. Zebrafish *in vivo* model system has many advantages such as inexpensive, easy to culture and breeding, and quick assay to display for teratogenic abnormalities (Westerfield, 2007). Frequently, the fish embryos and larvae have been used in toxicological tests of environmental pollution (Ceylan *et al.*, 2014). Thus, zebrafish is ideal for rapidly and economically testing car oil developmental toxicity. The toxicity of 10W-40 (unused semisynthetic) motor oil has not been previously studied in any animals. The current study used zebrafish embryos to explore developmental toxicity of 10W-40 motor oil in early life stage of zebrafish, including embryotoxic and teratogenic effects.

Materials and Methods

10W-40 semi-synthetic motor oil was obtained from commercial suppliers and set as water associated fraction (WAF). WAF of the motor oil was prepared according to Pauka *et al.* (2011). Briefly, the fraction was pouring 1 part of oil above 9 parts of reconstituted water in a bottle and slowly stirring the water at 20 rpm (revolution per minute) for 20 h at 23.0±2.0 °C. After mixing the oil and the water associated portion, designated the 100 v/v soluble fraction, were separated. Different concentrations (1.25, 2.50, 5.0, 10.0, 20.0 and 40.0 v/v) were prepared in flasks from the 100 v/v stock solution, and were used in the experiments. WAF motor oil concentrations were selected according to Pauka *et al.* (2011).

Adult fish (*Danio rerio*) were purchased from Atatürk University Fisheries Faculty, Research Center of Aquarium Fish. The fish were acclimated for two weeks before the experiments. Fish were housed at 20 fish per tank. Fish were maintained a 14:10 light/dark photoperiod. Dechlorinated water was kept on at 26±1 °C. The tank water was changed every week. The water was regularly tested. Fish were fed two times in a day with dry flakes. Dry flake food was fed twice daily and live food (*Daphnia*) was fed once every two days. Fish embryos were obtained from healthy adult fish. Two breeding groups were established separately in a spawning aquarium. Spawning was stimulated on the morning. After half an hour, the eggs of fish which free of macroscopically noticeable symptoms of infection and disease were collected, washed with Hanks' buffer embryo medium and incubated in petri dishes at 26±1 ^{o}C until the oil treatment was made. Hanks' buffer embryo medium was used as the control. Two hours of postfertilization (hpf) period, embryos in blastula stage were collected and selected under a stereomicroscope into a glass petri dish containing the medium. The incubation was started by addition of the fertilized eggs to the motor oil solutions at 2.5 hpf. Twenty embryos were placed each group. The each experiment was repeated three times. In total, 420 (20 embryos x 7 groups x 3 replicates) embryos were used for the experiment. The embryos were exposed in a glass petri dish (10 cm diameter) containing 50 ml test solution at 26±1 °C with a 14:10-h light/dark cycle in a sensitive incubator works under 72 hpf. The working solutions were replaced daily with prepared oil solutions.

The fish embryos were appraised and scored for lethal or sublethal effects using an inverted microscope (Nikon SMZ1000) equipped with a camera device (Nikon DSLR D70s) every 12 hours. Lethal malformations such as coagulation and missing heartbeat, somites, tail detachment, spontaneous movement were determined and dead embryos were ejected at each observation time. Sublethal malformations such as yolk sac edema, spine malformation, incomplete eye development, and no pigmentation were also reported. All embryos stages were described by (Kimmel *et al.*, 1995). Lethal, sublethal and teratogenic effects in embryos and larvae were recorded according to Lammer *et al.* (2009).

Malformation Index (MI) was conducted some modifications as previously described. Embryos and larvae were scored for malformation at 72 hpf by visual check under a dissection scope by rating scale of (Padilla *et al.*, 2011). According to the scale, it is assessed each embryo/larva for various categories (e.g. curved spine, abnormal head, edema). Each of the categories includes a number of possible malformations scored as yes/no or as degree. Scores from the each category were summarized to give a total MI for each embryo. MI values between 0-3 indicate normal; values between 4 and 6 indicate slightly abnormal, and values above 7 indicate obviously deformed fish embryo/larva. When the embryos asynchronously started to come out of the chorion, and the number of hatched embryos from 48

hpf was recorded. At 48 and 72 hpf, the number of larvae displaying morphological abnormalities were recorded. At 72 hpf, ten embryos were randomly selected from each experimental group to obtain mean body length with digital camera (Leica ICC50 HD camera) attached to microscope (Leica DM750).

Statistical analysis was performed with SPSS software programme (version 20.0). The results were reported as the average of three replicated samples. All of data was described as mean ± standard deviation (SD). One-way ANOVA was performed to determine statistical significance. The $p<0.05$ level was accepted for statistical significance in all cases.

Results

In the present study, the early life stage toxicity of various concentrations (1.25, 2.50, 5.0, 10.0, 20.0 and 40.0 v/v) of WAF 10W-40 motor oil was firstly determined. All of the test groups were compared to control group including Hanks' buffer embryo medium. The mortality of embryos after exposure to the oil was determined at 72 hpf (Table 1). There was a slight increase in the embryo mortality rates at 2.5 v/v. Approximately 12.2±1.92 of 60 embryos died in the 2.5 v/v of WAF the motor oil treatment group. However, the mortality increased sharply at concentrations of 5.0 v/v and above. When the oil concentration was 5.0 and 10.0 v/v, the mortality rates of embryo showed statistically significant according to control group at $p<0.05$. All embryos exposured to 20 and 40 v/v oil concentrations were died (Table 1). Approximately 90% of embryos seem normal in the oil concentrations 1.25 v/v and 2.5 v/v. The teratogenic effects of the embryos exposed to 5.0 v/v oil concentration were observed. In 10 v/v WAF the motor oil, the number of abnormalities increased to 88% at 72 hpf after the exposure (Table 2). The increasing were significantly higher than control in $p<0.05$ level.

Higher concentrations of WAF motor oil (≥10.0 v/v) caused acute lethal effects such as egg coagulation and non-formation of somites at 24 hpf (Figure 1A, B and C). The lower concentrations (≤5.0 v/v) caused several malformations at 24, 48 and 72 hpf, including incomplete gastrulation (IG) (Figure 1D), tail curvature (TC), weak pigmentation (WP), head malformation (HM) (Figure 2), pericardial edema (PE), vertebra column defect (VD) and scoliosis (S) (Figure 3).

The body length of live zebrafish larvae were measured at end of the period (72 hpf). The body length in treatment of 2.5 v/v and lower WAF oil concentrations didn't have a significant difference compared with the control. The 5.0% and 10.0 v/v groups reduced the body length of larvae ($p<0.05$) (Figure 4). In Figure 5, mean total Malformation Indexs (MI) for WAF motor oil concentrations 1.25 and 2.5 v/v were not statistically different from control embryos. However, WAF motor oil

Table 1. Mortality rates of the fish embryos at 72 hpf after exposure to different WAF motor oil concentrations

WAF motor oil concentrations (v/v)	**The rate of mortalities at 72 hpf**	**t-values**
Control	3.2±0.83	4.8
1.25	7.8±0.83	7.48
2.5	12.2±1.92	8.37
5.0	28.6±1.14*	46.28
10.0	41.4±2.70*	30.12
20.0	60.0*	-
40.0	60.0*	-

* Indicated a significant difference from the control at $P<0.05$ (n=60).

Table 2. Total lesions after exposure at different hours and specific lesions of embryos (72 hpf)

	Lesion rates (%)				
WAF motor oil concentrations (v/v)	24 hpf	48 hpf	72 hpf	Total	Specific Lesions
Control	2	1	0	3.5	IG (1), WP (1)
1.25	1	2	1	3.77	TC (1), PE (1)
2.5	3	4	3	10.63	IG (1), TC (1), WP (1), VD (2)
5.0	8*	12*	9*	29.03	IG (2), TC (1), WP (3), HM (1), PE (1), VD (2), S (2)
10.0	22*	46*	21*	88.23	IG (3), TC (3), WP (3), PE (1), VD (2), S (3)
20.0	0	0	0	0	None
40.0	0	0	0	0	None

*Indicated a significant difference compared from control at $p<0.05$. IG, TC, WP, PE, VD and S showed Incomplete Gastrulation, Tail Curvature, Weak Pigmentation, Head Malformation, Pericardial Edema, Vertebra column Defect and Scoliosis respectively. The number of abnormal embryos is indicated in parentheses.

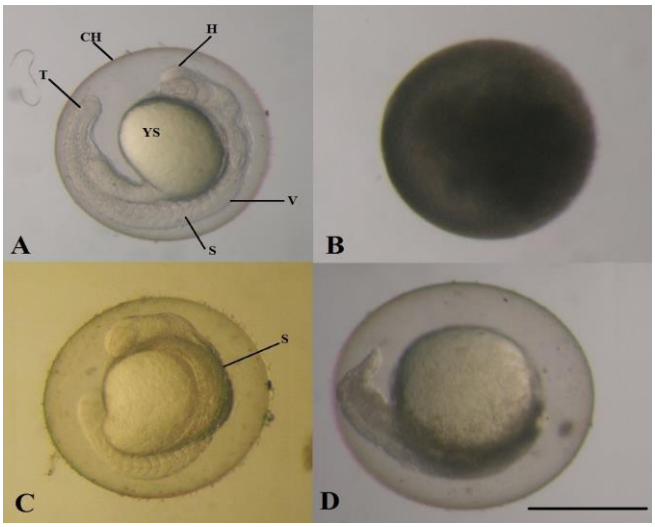

Figure 1. Acute lethal effects and sublethal effect in zebrafish embryos exposed to 10.0 v/v WAF motor oil. A) Normal embryo (24 hpf) with clearly somites, well-developed head, and tail. B) 24-hpf embryo exposed to car oil showing egg coagulation, C) showing non-formation of somites D) showing incomplete gastrulation. T; tail, CH; chorion, H; head, YS; yolk sac, V; vertebra, S; somite. Bar 500 µm.

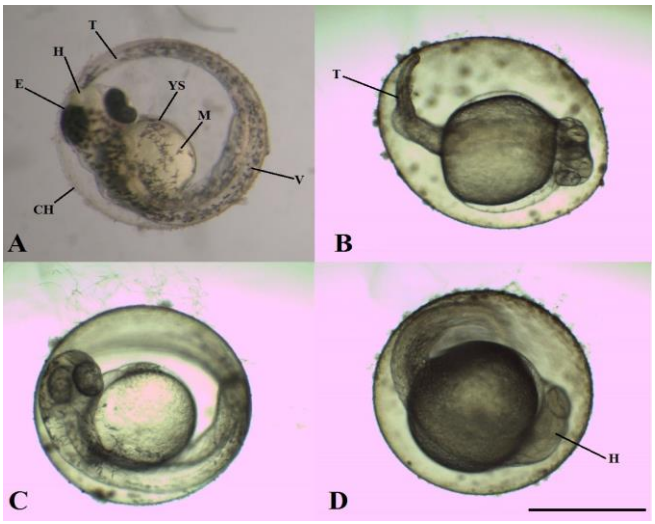

Figure 2. Some of malformaions in zebrafish embryos exposed to 5.0 v/v WAF motor oil at 48 hpf. A) Normal embryo with adequate pigmentation of retina and entire body. B) 48-hpf abnormal embryo showing tail curvature and C) weak pigmentation. D) 48-hpf abnormal embryo showing head malformation. T; tail, CH; chorion, H; head, E; eye, YS; yolk sac, V; vertebra, M; melanophores. Bar 500 µm.

concentrations of 5.0 and 10.0 v/v increased the mean total MI in 72 hpf embryos when compared with control. The 72 hpf embryos scored between 0 and 3 for MI were normal (control, 1.25 and 2.5 v/v); the 72 hpf embryos that scored between 4 and 6 were slightly abnormal (5.0 v/v); and he embryos scored above 7 were absolutely deformed (Figure 5). The hatching rate decreased with the increasing WAF motor oil concentrations at 72 hpf (Figure 6). The rates at 1.25 and 2.5 v/v concentrations of WAF motor oil didn't display differences in comparison with the control. Unlike, the hatching rates of the 5.0% and 10.0% groups were low, and showed delaying embryonic development and statistically significant toxicity. Also, no hatching was observed in 20 and 40 v/v WAF concentrations (Figure 7).

Discussion

The WAF of 10W-40 motor oil resulted in increased lethal effects and sublethal effects on zebrafish embryonal development. Lethality progressively increased from lower to higher concentrations of the WAF motor oil. The malformations of embryo-larvae and body length of larvae were the phenotypic endpoints to assess developmental toxicity of WAF motor oil. The overall results of the present study indicated that

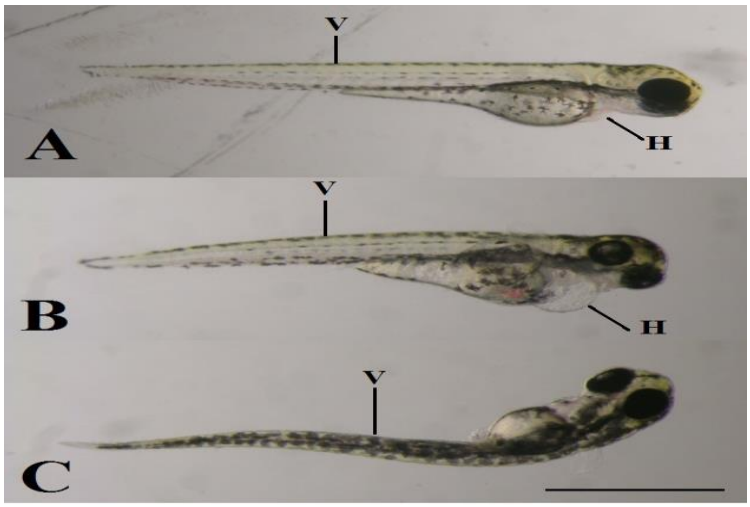

Figure 3. Sublethal effects in zebrafish embryos exposed to 5.0 v/v WAF motor oil at 72 hpf. A) Normal hatched larva with well-developed eye, pigmentation and normal body structure (lateral view). B) 72-hpf larva exposed to car oil showing pericardial edema and vertebra column defect (lateral view). C) 72-hpf larva showed scoliosis (dorsal view). H; heart, V; vertebra. Bar 500 µm.

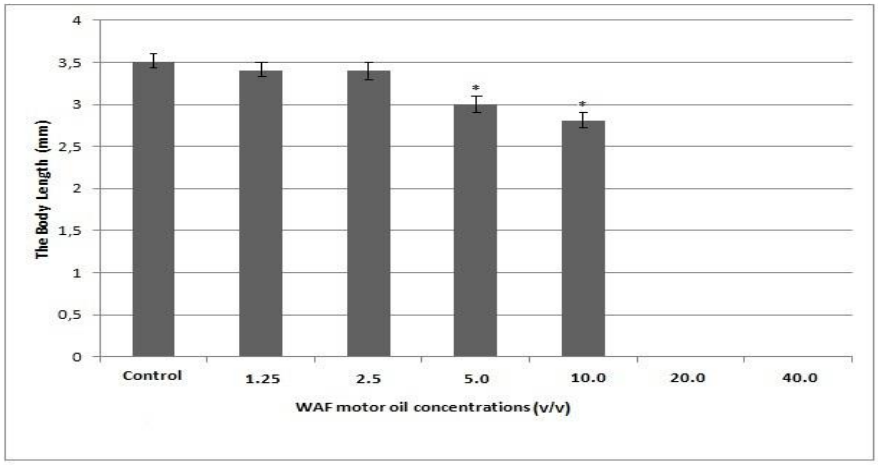

Figure 4. The body length of 72 hpf larvae after exposure to WAF motor oil concentrations. Asterisks indicated a significant difference when compared to control at P<0.05.

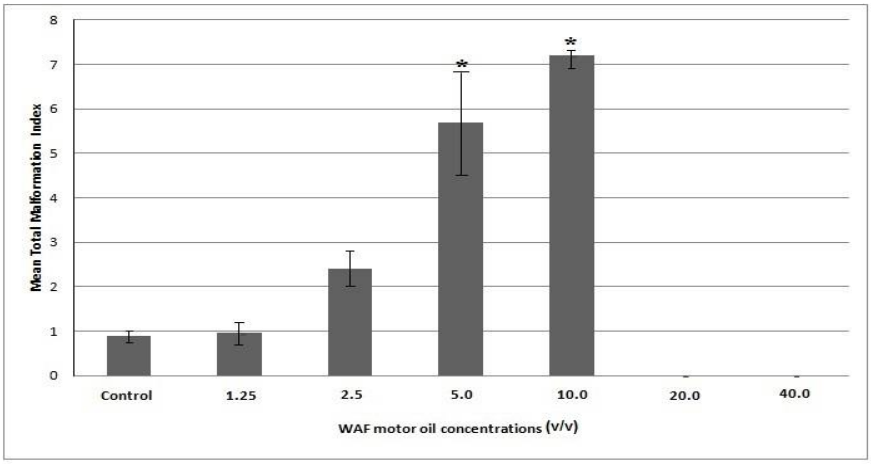

Figure 5. Total Malformation Index of the fish embryos exposed to WAF motor oil. Asterisks indicated a significant difference when compared to control at *p<0.05*. The number of live embryos: Control 57, 1.25v/v WAF 53, 2.50v/v WAF 48, 5.0v/v WAF 32, 10.0v/v WAF 19, 20v/v WAF 0, 40v/v WAF 0.

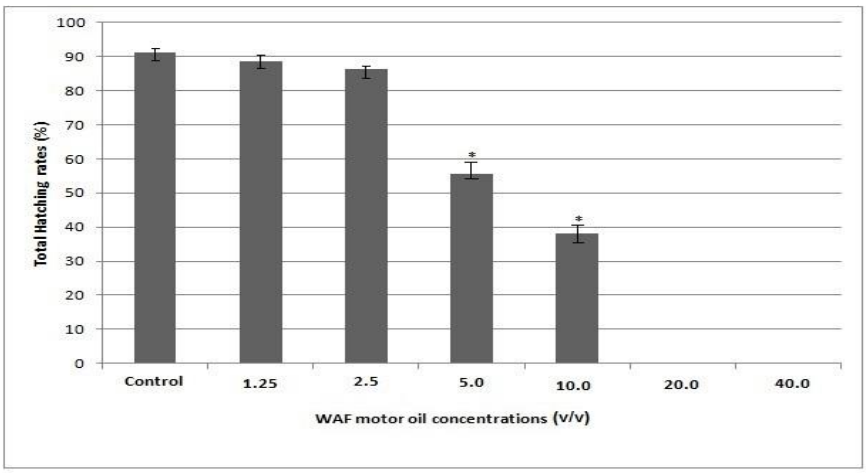

Figure 6. The hatching rates of zebrafish embryos after exposure of different WAF motor oil concentrations at 72 hpf. Asterisks indicated a significant difference when compared to control at P<0.05.

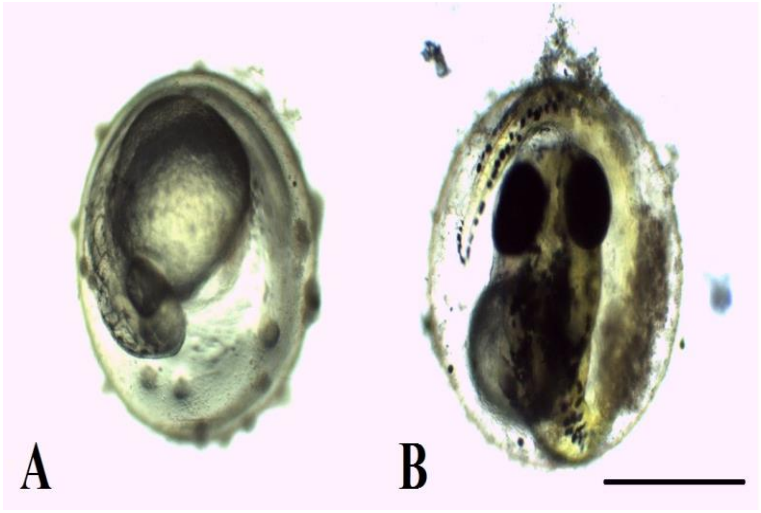

Figure 7. No hatching embryos at 48 hpf (A) and at 72 hpf (B) exposed to 20 and 40 v/v WAF motor oil concentrations. Bar 500 μm.

WAF motor oil affected the early life stages of zebrafish in dose-dependent manner.

The survival rate of embryos demonstrated that exposure to low WAF motor oil concentrations did not affect the survival during the test. However, the survival rate dramatically reduced in higher WAF motor oil concentrations. The oil reduced the embryos survival as depend on oil concentrations and was similar to those shown by the other studies. In a previous work, zebrafish embryos exposed to crude oil, and it was observed that fish embryos adversely affected at 15, 33 and 50 v/v crude oil concentrations. Also, a significant number of embryos died during exposure (Pauka *et al.,* 2011). Incardona *et al.* (2012) reported an important study on the issue. They investigated health and viability of *Clupea pallasii* embryos from oiled (polluted) and unoiled (clear) stations on San Francisco bay. They observed that in the fish embryos from the oiled location resulted in an increase lethality and necrosis. Likewise, markrecapture studies on *Oncorhynchus gorbuscha* following the Exxon Valdez spill (the last major US oil spill, 1989 Alaska) found that transient and sublethal exposures to crude oil during embryogenesis reduced subsequent marine survival to adulthood by 40 v/v (Heintz, 2007). Also, exposures to crude oil at high concentrations cause embryonic death (Incardona *et al.,* 2013). The current study is the first to report developmental toxicity of the WAF of 10W-40 motor oil to zebrafish, although embryotoxicity was previously reported for crude oil (Pauka *et al.*, 2011).

The WAF of 10W-40 motor oil resulted in increased lethal and sublethal effects on zebrafish embryos. Higher concentrations (≥10.0 v/v) caused acute lethal effects (egg coagulation, non-formation of somites), and lower concentrations (≤5.0 v/v) caused sublethal effects, such as incomplete gastrulation, tail

curvature, weak pigmentation, head malformation, pericardial edema, vertebra column defect and scoliosis. Moreover, with the increase of car oil concentration, the malformation rate increased. The hatching of embryos exposure to car oil delayed and body length of the larvae reduced. The results indicated that WAF 10W-40 motor oil affected the early life stage embryos of zebrafish a dose-dependent manner. Embryotoxic effects such as weak pigmentation, tail defects, and reduced heartbeat rate were reported in embryos exposed to 15, 33 and 50 v/v crude oil during 96 h (Pauka *et al.,* 2011). De Soysa *et al.* (2012) indicated that the Maconda crude oil WAF caused a various defects in zebrafish embryos. In the study, the zebrafish embryos were exposed to 100% WAF solution prepared from the 1:10 vortex-mixed stock solution, and some morphological deformations (severe cardiac edema, yolk sac edema, tail curvature, tail cysts) were observed. In several studies, the developmental toxicity of car/crude oil to aquatic vertebrates has also been reported. Some fish species embryos were exposured to crude oil and water soluble oil, and it was found that the oil caused cardiovascular abnormalities and pericardial edema in *Atherinops affinis* and *Clupea pallasi* embryos, as well as additional defects in jaw and spinal cord development in *Melanotaenia duboulayi* (Incardona *et al.,* 2009; Anderson *et al.,* 2009). Field and laboratory investigations demonstrated that there was a common syndrome of crude oil-induced developmental toxicity in a range of teleost fish. This was described by pericardial edema, body axis curvature, yolk edema and jaw reductions (Heintz *et al.,* 1999; Carls *et al.,* 1999; Pollino and Holdway, 2002; Couillard, 2002). These results of the studies were in agreement with the current study.

The mechanisms motor or crude oil-induced lethal and teratogenic effects during the fish development are unknown. The toxicity of crude oil has been reported by Overton *et al.* (1994) to be due to the water soluble fraction of the presence of toxic components like xylene, naphthalene, benzene and toluene. The water soluble fraction of crude oil and their derivatives products contains a mixture of polycyclic aromatic hydrocarbons (PAHs), monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes; phenols and heterocyclic compounds (Saeed and Al-Mutairi, 1999). A study showed that dissolved PAHs were toxic to zebrafish embryos. PAHs caused edema, developmental delays, hemorrhaging, and cardiac function abnormalities (Carls *et al.,* 2008). The target organ for crude oil is developing fish heart (Incardona *et al.,* 2011). These compounds directly disrupt fish cardiac function (Incardona *et al.,* 2005). It was observed that exposure of fish embryos to PAH mixtures derived from crude oil slowed the heartbeat and reduced contractility (Incardona *et al.,* 2013). The underlying mechanism was shown that it may be barricade of key potassium and calcium ion channels involved in cardiac excitation-contraction coupling (Brette *et al.,* 2014). These adverse effects of PAHs may influence the structure of fish body during embryonic and larval stages. In addition, it is known that some petroleum derived hydrocarbons are toxic to a wide spectrum of marine animals because they preferentially accumulate in lipidic compartments like cellular membrane, disturbing the physicochemical and physiological membrane properties (Di Toro *et al.,* 2001).

The present study is the first report to explore the developmental toxicity of WAF of 10W-40 motor oil in the early life stages of zebrafish, including lethal and sublethal effects. The overall results provide critical information for risk assessment of motor oil. The findings can be used as a guide to systematic analyses of native species that may have been affected by other lubricant oil.

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