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A comparative study on the effect of dispersed and undispersed Kuwait crude oil on egg hatching and larval survival of *Epinephelus coioides*

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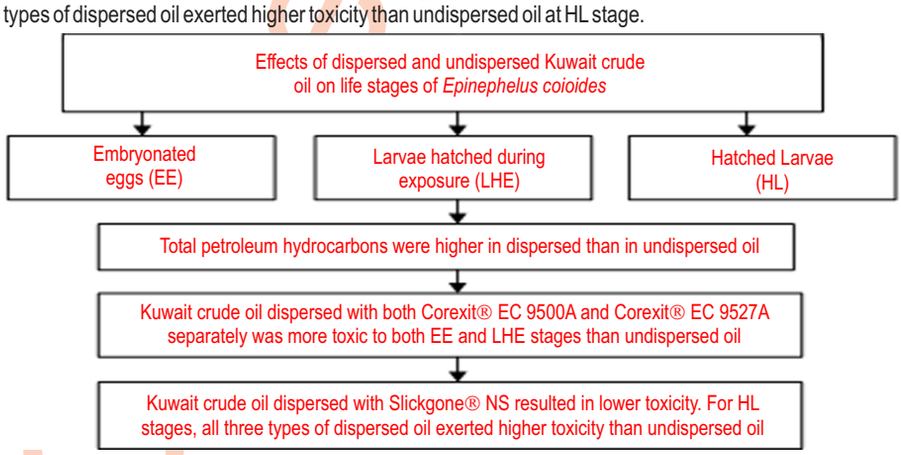
Abstract

Aim : The objective of the present study was to investigate the effects of dispersed and undispersed Kuwait crude oil on egg hatching and larval survival of *Epinephelus coioides*.

Methodology : In the present study, the toxic effects of crude and dispersed oil using three formulations of oil dispersants against multiple life stages of *Epinephelus coioides* was assessed. The lethal concentration was calculated by ToxCal® software developed by Tidepool Scientific, LLC.

Results : Specifically, the following life stages were investigated: embryonated eggs (EE), larvae hatched during exposure (LHE) and hatched larvae (HL). Chemical analysis showed that Total Petroleum Hydrocarbon (TPH) concentrations were higher in dispersed than undispersed oil solutions, indicating accommodation of more petroleum hydrocarbons in the aqueous phase. Acute static toxicity tests produced variable LC_{50} values for all chemical preparations and all fish life stages. Crude oil dispersed with both Corexit® EC 9500A and Corexit® EC 9527A separately was more toxic to both EE and LHE stages than undispersed oil, but crude oil dispersed with Slickgone® NS resulted in lower toxicity. Furthermore, all three types of dispersed oil exerted higher toxicity than undispersed oil at HL stage.



Interpretation : A life stage dependent effect demonstrated variation in the toxicity of both dispersed and undispersed crude oil to fish. Few life stages were more sensitive than others to either dispersed or undispersed crude oil toxicity. While dispersion of an oil slick with oil dispersant has proved to be an effective tool in the oil response strategy, the fate of dispersed oil can exert lethal effects on embryo-larval stages of marine fish present near the spill.

Key words: Embryo-larval stages, *Epinephelus coioides*, Kuwait crude oil, Total petroleum hydrocarbons

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Introduction

Oil spills affect aquatic life and their habitats in many ways (Birtwell *et al.,* 1999). The severity of the impact depends on the type and amount of oil spilled, season and weather, type of shoreline, and type of wave and tidal energy in the spill area (Martınez-Gomez *et al.,* 2010). Chemical substances and other related petroleum products are transported across global regions by ships or pipelines creating a possibility of spillage with the potential risk of environmental pollution (Daling *et al.,* 1990). In 1991, the Arabian Gulf experienced an oil spill accident which was estimated to be around 816,000 metric tonnes (Pearce, 1993; SOAFD, 1993; Wolf *et al.,* 1993).

Kuwait is one of the major oil-producing countries in the Arabian Gulf, and marine pollution is one of the most significant environmental issues with oil input from waste discharged is estimated to be 26,905 tonnes per year (UNEP, 1999). Spilled oil causes developmental abnormalities and mortality in zooplankton and early-life stages of other marine organisms (Afolabi *et al.,* 1985; NRC, 1985; Powell *et al.,* 1985; Otitoloju and Adeoye, 2003; Karam *et al.,* 2014). The toxicity of crude oil is, therefore, collectively due to the toxicity of organic and inorganic chemicals present, and its toxicity is further interpreted as the fraction of crude oil which can induce deleterious effects on marine fish species.

Oil dispersants are used for rapid removal of spilled oil from the sea surface and then dissolving oil into the water column. This can be performed by reducing the interfacial tension between oil and water and assist in the formation of minute droplets or mixed oil surfactant micelles which disperse in the water column where they can be diluted and biodegraded (ROPME, 1998). Dispersants are defined as chemical formulations that consist of individual components called surfactants, which possess two distinct oleophilic (oil-liking) and hydrophilic (water-liking) groups. Furthermore, the size of small oil droplets decreases due to dispersant, leading to an increase in the surface area exposed to water. These oil droplets determine oil toxicity (Ramachandran *et al.,* 2004; Brannon *et al.,* 2006), a result, the concentration of oil in the water column increases, thereby increasing the concentration of dissolved polycyclic aromatic hydrocarbons, which might result in fish toxicity (Canevari, 1978; ROPME, 1998; Couillard *et al.,* 2005). Although utilization of oil dispersants is an effective mean to combat oil spills in marine waters, there still exist numerous concerns about the toxic effect of dispersed oil on marine fish (Otitoloju, 2005; Venosa and Holder, 2007; Nyman *et al.,* 2007).

Many questions still remain unanswered about indirect toxicological effects of acute exposure to oil pollution and PAHs on the health of aquatic organisms (Bonsdorff *et al.,* 1990; Bejarano *et al.,* 2006). Orange-spotted grouper (*Epinephelus coioides*), locally named hamoor, is a key species in the aquatic ecosystem and an ideal model for toxicological study (Hussain *et al.,* 1981). It is one of the most highly priced seafood, and the demand of global markets demand exceed their supply, making orange-spotted grouper an economically important fish species around the world. In view of the above, this study mainly focused on the sensitivity of two different exposure regimes and to detect the dispersed and undispersed crude oil levels among the embryonated eggs and larval stages of *Epinephelus coioides.*

Materials and Methods

Epinephelus coioides was selected as a test species based on the criteria of ADEC (2000). The epinepheline serranids, form an important taxonomic group both from an ecological and commercial perspective (Sluka *et al.,* 2001; Sadovy de Mitcheson *et al.,* 2013). *E. coioides* is common in the Arabian Gulf where it is the most important commercially exploited species (Randall, 1995). This fish also forms an important component in the marine food web as their larvae feed on copepods and, along with other larval fish, are being preyed upon by chaetognaths and adult fish such as silver pomfrets (*Pampus argenteus*) (Baier and Purcell, 1997; Abdurahiman *et al.,* 2006; 2010). Different life stages, embryonated eggs, larvae hatched during exposure, and hatched larvae of *E. coioides* were obtained from the hatchery of the Aquaculture Program at Kuwait Institute for Scientific Research (KISR). The spawning period of this fish is from March to June; and this fish has been successfully cultured and extensively studied in Kuwait Institute for Scientific Research (KISR). The physico-chemical parameters of the water used in this study is as follows: dissolved oxygen: 5-6 $mg.l^{-1}$, temperature: 20-28°C, salinity: 40-42 ppt and pH 8.2-8.6.

Kuwait Export Crude Oil (KCO) (API-3.18) was procured from the Petroleum Research Center (PRC) of KISR and stored at room temperature (26°C) in dark. Kuwait Crude Oil technical specifications are as follows: gravity: 30.18 SG, density: 0.8744g ml^{-1} at 15ºC, sulphur content: 2.6% weight, viscosity: 17.38ºC at 20ºC and Conradson Carbon Residue: CCR 6.2 % weight. Chemical dispersants, Corexit® EC 9500A, Corexit® EC 9527A, and Slickgone® NS were procured from their original manufacturers Onedo Nalco Ltd. (2005), United Kingdom local agent Bobyan Shipping and Marine Services, and Dasic United Kingdom (2007) (local agent Middle East Chemical Manufacturing Co.).

Natural seawater was obtained from the near-shore wells and was filtered through a 0.45 µm Whatman® sterile membrane filter before being used for preparing water-accommodated fraction (WAF) and dilutions. One gram KCO filtered seawater used for preparing WAF, and 0.1g oil dispersant was selected and layered over the oil slick in a 2l glass aspirator bottle, for preparing chemically-enhanced water-accommodated fraction (CE-WAF). Crude oil and dispersants were layered over a known volume of filtered seawater, mixed for 24 hrs, and then left to stand for complete phase (oil/water) separation. WAF and CE-WAF solutions were drained, collected in amber bottles and preserved in a refrigerator until further use. A 96 hr acute toxicity tests were conducted following OECD Guidelines for the Testing of

Chemicals-Fish Embryo Toxicity (FET) Test (OCED, 2006). Solutions prepared from the following exposure chemicals were used: KCO alone (KCO WAF); KCO + Corexist® 9500 dispersant (Corexist® 9500 CE-WAF); KCO + Corexist® 9527 (Corexist® 9527 CE-WAF and KCO + Slickgone® dispersant (Slickgone® CE-WAF). Five serial dilutions of 100%, 50%, 25%, 12.5% and 6.25% were used to estimate the LC_{50} concentrations and 95% confidence intervals (Environment Canada, 1990). KCO WAF and KCO plus three individual oil dispersant (KCO CE-WAF) dilutions were made in 100 ml glass beakers and made up to a final volume of 50ml of exposure medium (WAF or CE-WAF). Serial dilutions were made in multiple replicates with non-toxic controls (only filtered seawater) for each replicate.

The acute static toxicity (non-renewal) test was conducted using the following fish developmental stages: embryonated fish eggs (EE) brought from the hatchery after 24 hr of their release; larvae hatched during exposure (LHE) from the same embryonated eggs exposed in the same test regime so that embryos and larvae of the same embryos were exposed for a total of 96 hr, and already post hatched larvae (HL) which were exposed for 96 hr to the test chemicals. Endpoints for the toxicity tests were: successful egg hatching at 48 hr, and mortality of either LHE or HL stages at 96 hr. Eggs of dead fish were counted at 24 or 48 hr, and dead fish larvae were counted at 24, 48, 72 and 96 hr, respectively.

Toxicity tests were terminated after 96 hr of exposure. Fish larvae were not fed throughout the exposure period. No feeding was required as the yolk sac nourishes fish larvae for three days and the oil globule further nourishes the same larvae for additional two days. EE were washed and checked for complete fertilization, and toxicity tests were carried out 8 hr post-fertilization. For HL, toxicity tests were initiated 24 hr post-hatching. Normally, the mass of an egg and larvae is about 0.75 and 0.10 mg, respectively. A minimum of 10 to 30 fish larva (EE or HL depending on availability) were placed in 100 ml glass beakers using a glass wide mouth Pasture pipette.

Analysis of total petroleum hydrocarbons was carried out by extracting 100 ml of WAF. CE-WAF solutions were extracted by adding MERCK® dichloromethane (CH_2Cl_2). The mixture was centrifuged, dried over MERCK® grade anhydrous sodium sulfate (Na_2SO_4) and glass wool, which were pre-soaked and then the solvent layer was withdrawn and collected. The collected extract was then analyzed by using an RF-5301 PC SHIMADZU® spectrofluorophotometer using 310 nm excitation and 360 nm emission wavelengths. The levels of TPH were calculated against a prepared standard multipoint calibration curve and reported regarding the Kuwait crude oil equivalents (MOOPAM, 1999).

Statistical analysis: Minitab® Statistical Software-Version 17© 2016, Minitab Inc. was used for conducting statistical analysis. A general linear model (GLM) was used to determine if the exposure concentration (%) and exposure time (hr) exerted a significant effect on fish egg hatching and larval survival during toxicity tests.The GLM functions on the premise and predicts one variable (dependent), In this study, it is the hatching or larval survival success response, from one or more variables (independent) like exposure concentration (%) and exposure time (hr). Lethal concentration, which affects 50% of the exposed fish population (LC_{50}), and the no-observed-effect concentration (NOEC) were calculated by ToxCal® software developed by Tidepool Scientific, LLC.

Results and Discussion

Chemical characterization of WAF demonstrated that as oil loadings increased, TPH in WAF solutions changed slightly and were not proportional to the increase in oil loadings, indicating that saturation of water-soluble compounds was achieved at 1g oil l^{-1} seawater loading (TPH= 2.2 mg.l^{-1}), and a further increase in oil content could not substantially increase partitioning of water-soluble compounds in the aqueous medium (Fig. 1.). Dispersion of crude oil with three oil dispersants separately resulted in variable TPH concentrations that reflected the ability of individual dispersant to solubilize some petroleum hydrocarbons in WAF more than other. Chemical analysis revealed that Corexit® 9500 CE-WAF had the highest TPH concentration (33.2 mg l^{-1}), followed by Corexit® 9527 CE-WAF (17.7 mg l^{-1}), Slickgone® NS CE-WAF (5.1 mg l^{-1}) and KCO WAF (2.0 mg l^{-1}) (Fig. 1.), respectively

Eggs of *E. coioides* exposed to 1g oil^{-1} seawater KCO WAF showed more than 90% hatching in most of the exposure concentrations after 24 hr exposure, and the same situation was observed with eggs in the control tanks during the same period of time. Hatching percentages were increased to 100% at 48 hr in most of the test concentrations, except at highest concentration where it decreased to 87%. Eggs that did not hatch at either 24 or 48 hr were considered dead. The mean 48 hr LC_{50} g oil l^{-1} seawater ± SD calculated from three replicates was >1.0±0.0/0.0 with a 95% confidence interval of 0.933-1.072. The effect of exposure time and concentration on egg hatching was not statistically significant (p>0.05), and only exposure concentration had a statistically significant effect (p<0.05). The NOEC was <1g KCO l^{-1}. *E. coioides* larvae (HL) showed 100% larval survival until 96 hr of exposure in controls, and at 3.12, 6.25 and 12.5% WAF/KCO dilutions. The survival of larvae was reduced by 2% and 53% at 25, 50% KCO WAF dilutions, respectively.

At the highest concentration, 13% of the eggs were un-hatched, the larvae that hatched did not survive, and all of them died by 96 hr of exposure. The average LC_{50} calculated for the three replicates of LHE at 24 hr was 1.035±0.06/0.035, 48 hr LC50 1.0±0.0/0.0, 72hr LC_{50} 0.92±0.21/0.12, and 96 hr LC_{50} 0.46±0.1/0.06 with a 95% confidence interval of 0.321-0.752 for 96 hr LC_{50} (Table 1). The effect of exposure time and concentration on LHE was statistically significant (p<0.05). The NOEC was 0.25g KCO l^{-1}. *E. coioides* larvae (HL) were exposed to serially diluted KCO WAF 1.0 g KCO l^{-1} seawater. In control, survival

Table 1: General Linear Model test showing the effect of oil and dispersed oil on variable life stages of *E. coioides* which was significant (p<0.05) and as determined by the lethal concentrations which affects 50% of exposed larvae (LC_{50}) (gl^{-1}) for Water-accommodated fraction (WAF) and chemically-enhanced water-accommodated fraction (CE-WAF's) of Kuwait crude oil (KCO) showing dispersed oil as being more toxic than KCO WAF for all life stages

Life stages	KCO WAF/ SD/CI	Corexit® 9500 CE-WAF/SD/CI	Corexit® 9527 CE-WAF/SD±/ CI	Slickgone® NS CE-WAF/ SD/CI	*p*
EE(48 hr LC_{50})	>1.0±0.0/ 0.933-1.072	0.53±0.13/ 0.356-1.49	0.17±0.055/ 0.128-0.229	2.34±1.69/ 0.453-2.147	*p<0.05*
LHE(96 hr LC_{50})	0.46±0.1/ 0.321-0.752	0.21±0.12/ 0.15-0.3	0.08±0.020/ 0.063-0.121	1.18±0.919/ 0.524-3.236	*p<0.05*
*HL(96 hr LC_{50})	0.93±0.77/ 0.465-0.917	0.015/0.0 13-0.018	0.01/0.08 9-0.0113	0.045/0.040 9-0.0498	*p<0.05*

EE= Embryonated Egg; LHE= Larvae Hatched during Exposure; HL= Hatched Larvae; LC_{50}= :Lethal Concentration affecting 50% of exposed fish; population; SD ±= Standard Deviation; CI= 95% Confidence Interval and ✻ = no SD only LC_{50} and CI

success was 97% during 24 hr exposure period, which decreased to 96% at 96 hr. The 100% concentration of KCO WAF exerted some toxic effects as the survival percentage was reduced from 100% at 24 hr exposure period to 21% at 96 hr. At lower dilutions, the minimal effect was observed as survival ranged from 87 to 99% until 96 hr exposure period. The average (LC_{50} g oil l^{-1} seawater ± SD) calculated from nine replicates after 24 hr was 1.26g KCO l^{-1} seawater for HL. This was comparably toxic to both 24 and 48 hr LC_{50} for the EE stages which were 1.075 and >1.0g KCO l^{-1} seawater, respectively. Conversely, the average LC_{50} values of nine replicates for HL larvae at 24 hr were 1.26±0.56/0.19, 48 hr LC_{50} 1.25±0.3/0.10, 72 hr LC_{50} 1.47±1.55/0.52, and 96 hr LC_{50} 0.93±0.77/0.25 seawater, with (0.465-0.917) 95% confidence intervals for 96 hr. The results demonstrated that HL showed more resistance (less toxicity) to KCO WAF than LHE during KCO WAF exposure (96 hr LC_{50} 0.468g KCO l^{-1} seawater). The effect of exposure time and concentration of 1g KCO l^{-1} seawater on survival success of larvae was statistically significant (p<0.05). The NOEC was <0.25g KCO l^{-1} (Fig. 2). It appeared that 1g KCO l^{-1} seawater loading could be more readily partitioned in seawater than 20g KCO l^{-1} seawater loading during KCO WAF preparation, which contributed more to its toxicity against *E. coioides* larvae.

The present study, to our knowledge, is the first report on the subsequent fate of early life stages of *E. coioides* exposed to crude oil and dispersed oil. Fish toxicity data are highly variable due to several factors such as maturity, species and size that govern the overall sensitivity of a test system. Different fish species and life stages have variable responses to the toxic effect of dispersed and un-dispersed crude oils (NRC, 2003). Early life stages of fish tend to be most sensitive to crude oil exposure, and comparison of toxicity results among test species and their life stages, and the types of toxicants investigated are complex, if not impossible,because there are significant differences in methodologies used to generate valid data (Shales, 1989; Norcross *et al.,* 1997; Singer *et al.,* 2000). The life stages of *E. coioides* responded differently to KCO WAF, and it was found to be more toxic to larvae hatched during exposure, than hatched larvae, or embryonated egg stage which was the most resistant stage to toxic effect. In several studies, a discrepancy in sensitivity to hydrocarbons between developmental stages has been observed; a fish larvae and young fry are more sensitive to water-soluble fractions than eggs (Kunhold, 1970; Struhsaker *et al.,* 1974; Moles *et al.,* 1979).

In contrast to the present findings, Raimondo *et al.* (2014) observed developmental defects and cardiotoxicity in zebrafish (*Danio rerio*) embryos exposed to sediments contaminated with South Louisiana crude oil. Barron *et al.* (2004) indicated that the toxicity of WAF and CE-WAF solutions were similar in exposed fish eggs and larvae,while other studies have demonstrated mixed responses and a decreased toxicity of CE-WAF solution in comparison to WAF (Pollino and Holdaway, 2003; Gagnon and Holdaway, 2000; Wheelock *et al.,* 2002; Georgiades *et al.,* 2003). In contrast to our study, Carls *et al.* (1999) observed that exposure of herring fish to oil increased in polycyclic aromatic hydrocarbons concentration in the fertilized eggs, but no considerable negative effects were noticed at the early stages of life .

The effect of CE-WAF mixtures on fish species examined was variable, resulting in a species and life stage dependent effect. Also, when living organisms are simultaneously, exposed to two or more chemicals the specific interaction between the constituents may result in an enhanced ultimate effect of the toxic chemicals (Cluevers, 2003; Otitolujo, 2003; 2005; Samuel *et al.,* 2008). Exposure analysis of Kuwait crude oil water-accommodated fraction (KCO WAF) revealed that (Table 1) the 48 hr LC_{50} for embryonated egg stage was >1.0±0.0 g l^{-1}, for larvae hatched during exposure the 96 hr LC_{50} was 0.46 g l^{-1} c, and for hatched larvae the 96 hr LC_{50} was 0.93 g l^{-1}. For chemically-enhanced water-accommodated fraction of Kuwait crude oil (KCO CE-WAF), EE 48 hr LC_{50} for Corexit® 9500 CE-WAF was 0.53 g l^{-1}, for LHE the 96 hr LC_{50} was 0.21 g l^{-1}, and for HL the 96 hr LC_{50} was 0.015 g l^{-1}. For Corexit® 9527 CE-WAF, the 48 hr LC_{50} for

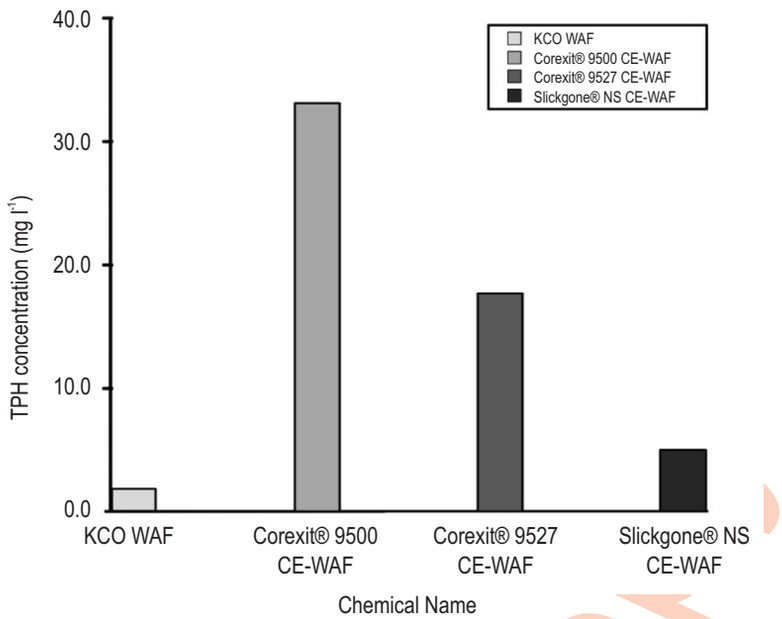

Fig. 1: Total Petroleum Hydrocarbons for KCO WAF (Kuwait Crude Oil Water-Accommodated Fraction) and CE-WAF (Chemically-Enhanced Water-Accommodated Fraction) of Kuwait crude oil with different dispersants (Corexit® EC 9500A, Corexit® EC 9527A and Slickgone® NS).

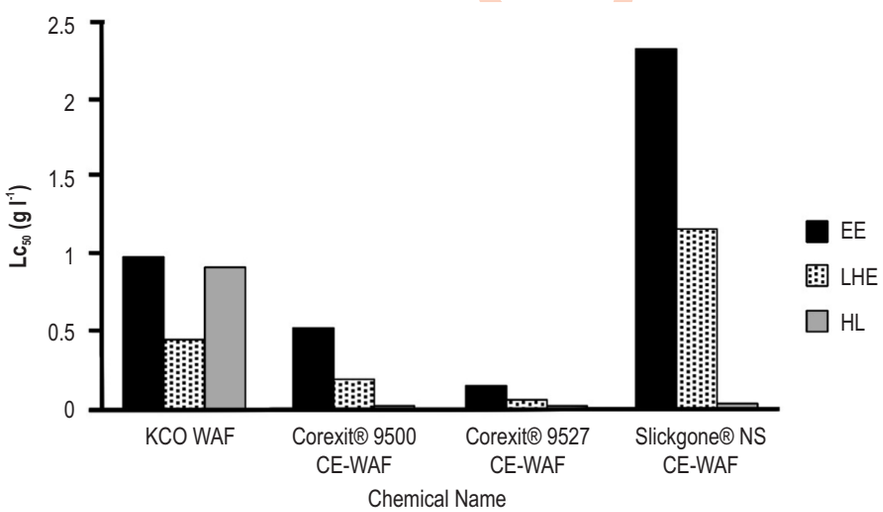

Fig. 2: Effect of total petroleum hydrocarbon (TPH) concentrations (mg l^{-1}) for KCO WAF (Kuwait Crude Oil Water-Accommodated Fraction) and CE-WAF (Chemically-Enhanced Water-Accommodated Fraction) of Kuwait crude oil with different dispersants (Corexit® EC 9500A, Corexit® EC 9527A, and Slickgone® NS on embryonated eggs (EE), larvae hatched during exposure (LHE), and post hatched larvae (HL) with LC_{50} at each exposure.

EE was 0.171 g l^{-1}, for LHE the 96 hr LC_{50} was 0.087 g l^{-1}, and for HL the 96 hr LC_{50} was 0.010 g l^{-1}. Finally for Slickgone® NS CE-WAF, the 48 hr LC_{50} for EE was 2.34g l^{-1}, for LHE the 96 hr LC_{50} was 1.185g l^{-1}, and for HL the 96 hr LC_{50} was 0.0452g l^{-1} respectively. The toxicity pattern observed for the EE life stage of *E. coioides* showed that Corexit® 9527 CE-WAF was the most toxic test chemical examined and dispersing KCO with Slickgone® NS was not more toxic than KCO WAF alone. The findings of cohen and Nugegoda (2001) indicate that exposure of fish to Bass Straight crude oil treated with Corexit® 9527 dispersant resulted in more toxic medium than oil WAF alone without

adding dispersant to it which is similar to the present findings in case of Corexit® CE-WAFs. Clark *et al.* (2001), on the other hand, observed that KCO dispersed with Corexit® 9527 was more toxic to turbot (*Scophthalmus maximus*) and inland silverside (*Menidia beryllina*) embryos and larvae as compared to the present study.

The resistance of fish eggs to oil toxicity was probably due to the presence of egg envelope (chorion) caused by the presence of enzyme transglutaminase (Tgase). Coupled with the vitelline membrane, the chorion protects the developing embryo from external chemical, physical and biological stressors in the marine environment, and also provide some defense mechanism against xenobiotic chemical intoxication (Yamagami *et al.,* 1994; Ha and Luchi, 1998; Finn, 2007). Exposure of LHE stage to KCO WAF and CE-WAFs produced a similar toxicity pattern to what was observed for EE stage.

Successful hatching of fish eggs during exposure to KCO WAF and CE-WAF indicated the resistance of EE towards toxicity. Although, the LHE stage lacked the protection of chorion egg membrane and, at hatching, they became more susceptible to KCO WAF and CE-WAF toxicity, the toxicity pattern was similar to that of EE stage. Previous studies have reported that during early development, damage to few precursor cells resulted in damage to the exposed fish (Greene *et al.*, 2007). The mechanism by which dispersants alter the hydrocarbon bioaccumulation process is not well understood (Mielbrecht *et al.,* 2005).

In this study, KCO CE-WAF caused a sharp decrease in 24 hr LC_{50} values compared to KCO WAF, indicating quick enhancement of toxicity of KCO by treatment with dispersants. At 48 hr, the LC_{50} values of WAF and CE-WAF were comparable, and with time the severity of toxicity increased with both WAF and CE-WAF. The toxicity pattern observed for HL showed that Corexit® 9527 CE-WAF was the most toxic chemical, and KCO WAF was least toxic and Corexit® 9527 CE-WAF toxicity value was close to that of Corexit® 9500 CE-WAF. Singer *et al.* (2000) demonstrated that the primary function of oil spill dispersant was to increase the entry of oil into water column, thereby modifying the exposure medium and increasing its toxicity.

Dispersion of crude oil with oil dispersant (CE-WAF) increased its toxicity in comparison to that of KCO WAF, as dispersants solubilized more of the oil fraction in the water column which rendered it bioavailable to fish larvae (Singer *et al.,* 1998). Jung *et al.* (2009) reported that addition of dispersants to crude oil enhance the concentration of hydrocarbons available to ovoviviparous rockfish (*Sebastes schlegeli*), since cytochrome P450-1A and EROD activity increased in the fish after exposure to crude oil WAF after dispersion with the dispersant Corexit® 9500. (Farid *et al.,* 2016) The results of this study corroborates the findings who reported that dissolved crude oil exerted more effects on hatched larvae than on embryos, and dispersed oil was more toxic to fish larvae than floating oil. The change in the order of toxicity of CE-WAF mixtures may be related to different degradation rates and degradation products of the dispersant, indicating that toxicity data vary for different oil dispersants and crude oil (Pollino and Holdaway, 2002).

In conclusion, the results of the study revealed that the total petroleum hydrocarbons concentrations were higher in dispersed than undispersed oil solutions, which shows that the accommodation of more petroleum hydrocarbons in the aqueous state.

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