Effect of Water-Accommodated Fraction of Kuwait Crude Oil on Developmental Stages of Orange-Spotted Grouper Hamoor (*Epinephelus coicoides*)

Q. Karam, M. U. Beg, A. Al-Khabbaz, Z. Al-Ballam, S. Dakour, and K. Al-Abdul Elah

Abstract - Oil pollution in the marine environment is one of the major concerns of the Arabian Gulf States and it can exert a variety of harmful effects in marine organisms and is known to disrupt the embryonic development in fish. Therefore, the lethal and sublethal effects of water-accommodated fraction of Kuwait crude oil (KCO WAF) were examined on embryonated eggs and the larval stages of hamoor (*Epinephelus coicoides*). The calculated 96h LC_{50} was 0.48g oil/l seawater. WAF of KCO produced no adverse effect on hatching of hamoor embryontaed eggs, whereas larvae exposed to the same medium were affected and most of them died by 96-h of exposure at 0.5 and 1.0g KCO/l of seawater. Maximum occurrences of deformity were also found in larvae at these concentrations. The types of deformities were type-A (lordosis-inward curvature of spine or V-shaped) which was followed by type-C (scoliosis-lateral bending of spine), then type-B (kyphosis- hunchback). The types of deformities resembled the reported deformities in literature which can be linked to the presence of polycyclic aromatic hydrocarbons (PAHs) in WAF of KCO. These responses can be used as a bio-indicator of petroleum hydrocarbon pollution in the marine environment.

Keywords---Embryos, Fish, Larval Deformity, Toxicity Water-Accommodated Fraction

I. Introduction

THE Arabian Gulf receives 2 to 4 million barrels of crude oil each year, mainly due to the shipping of oil. Over 30% of the world's marine transporters of oil cross the waters of the Gulf to load crude oil from the 25 major oil terminals located in the coastal areas. Some oil components such as lower aromatics, paraffins and nonhydrocarbon constituents are soluble in water; and while oil spreads over the surface of the sea, it gets dissolved into water rapidly.

Thus, water accommodated fraction (WAF) of crude oil represents highly volatile and toxic mixture of hydrocarbon chains like polyaromatics, phenols, and heterocyclic compounds containing nitrogen and sulfur. The components of WAF are considered as an important determinant of the petroleum toxicity in accidental spills [1]. Fish can quickly absorb a part of the volatile, mostly BTEX compounds (monocyclic aromatic hydrocarbons, which consist primarily of benzene, toluene, ethylbenzene and xylene) in WAF with adverse consequences to biological organization [2], [3].

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Polycyclic aromatic hydrocarbons (PAHs) are the second most abundant class of toxic compounds known for their carcinogenic potential in oil after BTEX [4].

All crude oils contain compounds toxic to marine organisms, some form extensive, and widespread slicks, and others settle on the bottom and incorporate large amounts of sand in globules. Spilled oil on the surface of water bodies exerts harmful effects, because it limits gaseous exchange, entangles and kills surface organisms, and coats the gills of fish [5], [6]. It also depresses phytoplankton photosynthesis, respiration and growth, kills or causes developmental abnormalities in zooplankton and the young stages of many aquatic organisms, and causes tainting of fish, shellfish, etc. [7-10]. The majority of fish produce large number of floating eggs. These and the larvae which hatch from them, drift in the surface currents. Therefore, early life stages of fish are considered to be the most sensitive life stages to oil spills and other environmental factors. Environmental factors can affect the early stages of development of the fish larvae such as exposure to pollutants. Among pollutants, a variety of xenobiotics ranging from trace metals to pesticides and petroleum hydrocarbons exert harmful effects on the early life stages of fish. The pollutants can produce various types of larval deformities like vertebral, craniofacial, cardiovascular, and yolk sac deformities accompanied by edema and hemorrhage. Acute toxicity experiments utilizing early life stages of fish are often used to determine legally applicable measurements of pollutants and to measure their effects on aquatic biota [11]. Although frequent oil spill studies are not available on the toxic effects of oil and its component to the early life stages of fish or any other biota. A possible reason was due to the mortality of eggs and young stages associated with oiling of spawning and nursery grounds [12]. The WSF of crude oil contains a mixture of PAHs, phenols, and heterocyclic compounds containing nitrogen or sulfur [1].

Although the more toxic compounds are volatile, fish can quickly absorb part of the WSF with adverse consequences to biological organization [2]. Tidal action could force interstitial water past oil-contaminated sediment toward the intertidal area where embryos absorb PAHs from transported water. Developing embryos contain a lot of lipids and can act as lipid packets which accumulate PAHs from a low level of exposure. The bioavailability of the PAHs to embryos, and the larvae of pink salmon absorbing PAHs across the yolk membrane of eggs and exposure for months to levels as low as 1.0 µg/l was found to be toxic [13]. Larval exposure to 1.0 µg/l of benzo-a-pyrene can lead to heritable reductions in egg viability of

fathead minnows [14]. Larvae of the different species exposed to phenanthrene and pyrene show slight toxic responses observed as malformation (bilaterally bent chord) toward the end of the exposure period [15]. Early life-stage toxicity to rainbow trout exposed to 9 to 34 µg/l of the C_4 alkyl phenanthrene (1-methyl-3-isopropyl phenanthrene; retene), including hemorrhages (yolk sac, pericardial sac, ocular and cranial tissues), yolk sac edema, skeletal deformities, and mortality [16]. The effects of seven nonalkylated PAH compounds concluded that larvae treated throughout embryogenesis with naphthalene, anthracene or chrysene have grossly normal anatomic features. In contrast, larvae treated with fluorine, dibenzothiophere, or phenanthrene display dorsal curvature of the trunk and tail and significant growth reduction partially of the head. In addition, dibenzothiophene and phenanthrene-treated embryos show severe pericardial and yolk sac edema, while fluorine treatment resulted in mild pericardial edema.Treatment with pyrene resulted in mild pericardial edema and a less-pronounced dorsal curvature, and other distinct defects [17].

The objective of this study was to assess the effect of KCO WAF exposure on orange-spotted grouper hamoor (*Epinephelus coicoides*) fish embryonated egg hatching and the survival of larvae, in addition to gathering a detailed visual evidence of the types of deformities in eggs and larvae upon exposure to KCO.

II. Materials And Methods

A. Preparation WAF of KCO

1. Preparation of Water Accommodated Fraction

Kuwait export crude (API-3.18), a blend of oil produced in Kuwait, was procured from the Petroleum Research Division of Kuwait Institute for Scientific Research (KISR), stored in a 2-l amber bottles with no head space. Kuwait crude oil water-accommodated fraction (KCO WAF) was prepared using a single oil: water (1:10) loading; and subsequently serial dilution of WAF was done for the fish toxicity exposure [18]. The technical specifications of this crude oil constituted the following: Gravity (30.18º), density (at 15º was 0.8744 g/ml), sulphur content (2.6 % weight), viscosity (at 20º 17.38 cSt), salt (< 0.1 bpb), and Conradson Carbon Residue (CCR- 6.2 % weight). Natural seawater was filtered through 0.45-µm filter and characterized for water quality parameters such as: pH 8.16, conductivity 62.3 ms/s, salinity 39.9 ppt, temperature 23.2°C, and dissolved oxygen 13.4 mg/l). The water was taken in a 2-l aspirator bottle, and a Teflon covered magnetic bar was placed in each bottle and it was then placed on the magnetic stirrer plate. The oil was layered gently from the top over the water in the aspirator bottle and stirred for 24 h. Then, it was left undisturbed for 30 min to separate into two layers of the fluid. The lower layer of WAF was siphoned in a conical flask and stored in 2l amber bottles until exposure time.

2. Chemical Characterization of KCO WAF

The WAF of Kuwait crude oil was analyzed for total petroleum hydrocarbon (TPH), BTEX, and PAHs. TPH was determined by Fourier Transform Spectroscopy (FT-IR) Analysis according to EPA procedure [19] with some modifications made. The WAF was extracted with CCl_4 and the TPH in the collected extracts was quantified by using the FT-IR instrument. WAF was extracted with dichloromethane, cleaned and reduced for the estimation of PAHs by the gas chromatography/mass spectrometry (GC/MS) method. BTEX compounds were directly estimated in WAF using a closed-system, purge-and-trap method by GC/MS [20].

B. Toxicity Testing

1. Acute Toxicity Tests

Acute lethality test method [21] was followed to conduct fish toxicity test. The exposure was conducted for 96 h starting from fertilized eggs; hatching took place in 24 h, and the larvae were exposed for three days under the test protocols. It was considered that yolk sac nourishes the larvae for three days, and oil globule nourishes for another two days; therefore, feeding was not recommended during the 96-h bioassay.

After collection and washing of fertilized eggs, exposure to WAF representing different amount of oil loadings on seawater was carried out separately for eggs and freshly hatched larvae. In 100-ml glass beakers, 50 ml of WAF prepared at different oil loading and the control (only seawater) was taken. Initially a range finding test with log doses was done for dose selection for toxicity testing; and subsequently, dose interval factors were narrowed down. A preliminary experiment conducted in the laboratory showed that the WAF prepared at 1-ml KCO loading per liter of seawater provided 100% mortality in 96-h test for egg hatching and survival of larvae. A minimum of 15-20 eggs were placed in control, and WAF containing beakers and the exposure were continued for 96 h. The weight of an egg and larvae were about 0.75 and 0.10 mg, respectively, and experiments run with several controls at this biomass loading provided satisfactory survival rates. Exposure regimens were initiated at times ranging from 4-to 8-h post fertilization (hpf). Results of acute toxicity tests were calculated in terms of an LC_{50} (median lethal concentration) at the end of the test, and information concerning the dependence of adverse effects on both time and concentration were also collected. The LC_{50} value for each day of exposure was determined [22].

2. Morphological Abnormalities

During exposure period, the eggs and larvae were examined daily for the malformations under the stereomicroscope with a high magnification. Malformation or abnormality observed was photographed for documentation and scoring. Alterations of growth or development were mapped for spinal deformation, yolk sac deformity or edema, fin fold, head and body size. Deformity types have been classified as follows: Type-A- Lordosis (V-Shaped), Type-B-Kyphosis (hunch back), and Type-C-Scoliosis (Sideway).

C. Statistical Analysis

All statistical analyses were conducted using General Linear Model (GLM) to determine whether or not exposure concentration (%) and exposure time (h) exerted a significant effect on fish egg hatching and/or larval survival during the

toxicity test [23]. Test of significance was conducted, p values were calculated, and effects were considered significant if ($p<0.05$). The initial estimation was performed with Spearman-Karber parameter estimation [24].

III. Results

A. Chemical Analysis of WAF

The WAF prepared at 1.0 g KCO/l seawater was chemically characterized and the data were collected for BTEX and TPH in WAF of KCO. BTEX concentrations were 0.04, 0.10, 0.10, and 0.06 mg/l, for benzene, toluene, ethylbenzene, and xylene respectively, while TPH was 2.22 mg/l and ∑PAHs 22.0 ng/l.

B. Toxicological Assessment

Hatching success of hamoor eggs in control seawater was conducted in repeated experiments before exposure to WAF in seawater alone under laboratory conditions. The average hatching success of hamoor eggs in 15 control experiments with seawater alone was 96% at 24 h, with most of the controls achieving more than 90% hatching. Some eggs which did not hatch at 24 h, eventually hatched by 48 h, achieving 100% hatching. The hatched hamoor larvae were healthy, and no malformation was noticed (Table I).

When exposed to WAF of KCO above 90%, the hatching of eggs was observed in most of exposure concentrations at 24-h exposure period, similar to that found in controls. The hatching percentage was increased to 100% at 48-h, except at the highest exposure concentration where some of the hatched larvae died to reduce survival to 87% at 48-h exposure period. Eggs which did not hatch at either 24 or 48 h were considered dead beyond this point. The calculated 48-h LC_{50} was 1.13-g KCO/l seawater with (95% confidence interval 0.23 to 5.51).

The larvae which hatched during exposure to WAF showed 100% survival until 96 h of exposure in controls and at 12.5, 6.25 and 3.12 % dilutions of WAF of KCO. Increase in exposure concentration to 25% WAF caused 2% mortality, and at 50% WAF 53% larvae died, whereas at 100%, WAF all the exposed larvae died at 96h (Table II). The calculated 96-h LC_{50} was 0.48 g KCO/l seawater with (95% confidence interval 0.45 to 0.53). The effect of exposure time and concentration of 1-g/l KCO oil loading was statistically significant ($p<0.05$).

C. Abnormalities Assessment

In exposed hamoor, eggs and larvae deformity were determined using a stereomicroscope and deformity types were categorized. Most of the eggs exposed to WAF hatched successfully during the 24-h period. Eggs which were not hatched by 48 h found dead. The egg stage appeared to be more resistant to the toxic action of KCO-WAF which was reflected in the successful hatching process. Larvae hatched during exposure seemed to be vulnerable to the WAF toxicity and showed specific deformity types which reflected the stress induced by the toxic effect of crude oil on the overall health status of each larva, compared to the healthy control larvae which did not exhibit any deformity. In the exposed larvae, mainly Type-A (Lordosis-inward curvature of spine or V-shaped), Type-B (Kyphosis-hunchback), and Type-C (Scoliosis-Lateral bending of spine) deformities were observed as shown in (Fig. 1,2 and 3).

TABLE I
PERCENTAGES and AVERAGES OF SOBAITY 48-h LARVAL HATCHING and 96-h SURVIVAL SUCCESS for CONTROL SEAWATER

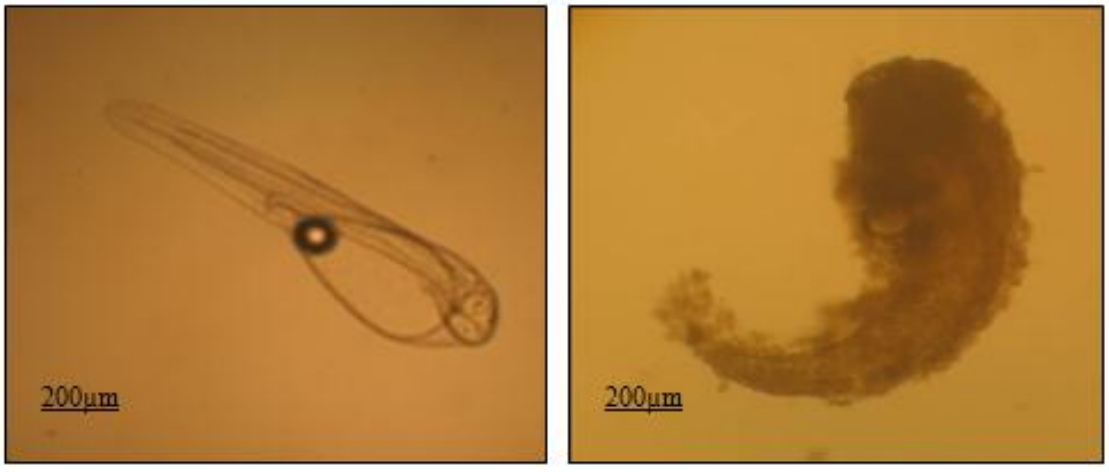
	No. of Controls														
Exposure Time (h)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0 h	16	20	16	16	15	15	19	18	16	17	10	10	10	10	11
24 h	16	18	16	15	14	15	19	18	16	16	8	10	10	9	11
24-h Hatching Success(%)	100	90	100	94	93	100	100	100	100	94	80	100	100	90	100
48 h	16	20	16	16	15	15	19	18	16	17	10	10	10	10	11
48-h Hatching Success (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
72 h	16	20	16	16	15	14	19	18	16	17	10	10	10	10	11
72-h Survival Success (%)	100	100	100	100	100	93	100	100	100	100	100	100	100	100	100
96 h	16	20	16	16	15	14	19	18	16	17	10	9	9	9	10
96-h Survival Success (%)	100	100	100	100	100	93	100	100	100	100	100	90	90	90	91

TABLE II
HATCHING SUCCESS of HAMOOR EGGS and LARVAL SURVIVAL AFTER EXPOSURE WAF of KCO[f] and CONTROL

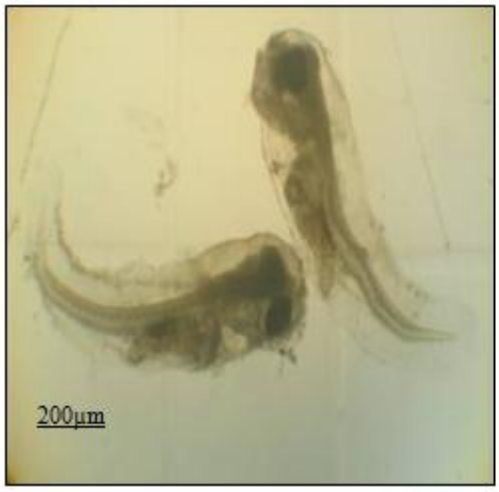
	WAF Concentrations (%)						
Exposure Time (h)	Control	3.12%	6.25%	12.50%	25%	50%	100%
0 h Total	45	45	44	47	43	47	52
24-h Hatching Success (%)	100%	96%	100%	98%	100%	89%	96%
48-h Hatching Success (%)	100%	100%	100%	100%*	100%	100%**	87%**
72-h Survival Success (%)	100%	100%	100%	100%	100%	98%**	27%**
96-h Survival Success (%)	100%	100%	100%	100%	98%**	47%**	0%**

*: Denotes that all the eggs hatched by 48 h.
**: Denotes that this % of larvae have survived.
[f]: Denotes that WAF was prepared by 1.0g KCO/l seawater.

(a) Healthy hamoor egg. (b) Dead hamoor egg.
Fig. 1 (a) Healthy hamoor eggs in control seawater , and (b) Dead hamoor egg in control



(a) Healthy hamoor larva. (b) Dead larva.
Fig. 2 (a) Healthy hamoor larva in control seawater, and (b) Dead hamoor larva



(a) Type C deformity- Scoliosis (left) and Type A deformity- Lordosis (right)

(b) Severe Type-A Deformity

(c) Type-B Deformity Kyphosis-hunchback

Fig. 3 Representing different deformity types for hamoor Larvae (a) Type-C deformity- scoliosis (left), and (b) Severe Type-A deformity and Type-A deformity- lordosis(right), and (c) Type-B deformity kyphosis- hunchback

In three separate exposure experiments to serially diluted KCO-WAF prepared at 1.0g KCO/l loading, the occurrences of deformity types indicated that deformities in some larvae appeared at 48 h of exposure with the majority of deformed larvae in the 100% exposure concentration. In exposure I, out of 26 eggs exposed, 19 larvae were deformed with Type-A deformity and only one with Type-C, and most deformed larvae died by 72 h. At 50% concentration, four larvae had Type-A deformity at 48 h and deformity increased to eleven surviving larvae at 72 h. At 96 h, only one deformed larva survived. At 25% WAF exposure concentration, also Type-A deformity was noticed in one larva which died at 96 h, and 13 larvae survived out of 14 initially exposed (Table III). In a second exposure experiment, deformities observed in 100% concentrations were of types A, B, and C. At 50% WAF concentration, only Type-A deformity was noticed, as observed in the previous exposure experiment. At lower dilutions, no deformity of any type was observed (Table III). In the third exposure experiment, the response of lower magnitude was observed. Only Type-A deformity was noticed in 50 and 100% exposure concentrations, in which around 30% larvae were deformed by 72 h (Table III). Exposure to WAF showed that increasing time and concentration increased the frequency of deformity and decreased survival of deformed larvae. At 50% WAF concentration, most of the larvae hatched by 48 h were deformed showing Type-A deformity. Which struggled to survive by 72 h, but died in mass by 96 h leaving only 7% of surviving larvae. At 100% WAF concentration, hatched larvae were deformed showing mostly deformity of Type-A; but other types of deformities like Type-B and Type-C were also found. At this exposure concentration most of the deformed larvae died at 72 h.

TABLE III
DEFORMITY TYPE PERCENTAGES OF HAMOOR LARVAE AFTER EXPOSURE KCO WAF[f]

	WAF Concentration (%)					
Exposure Time (h)	3.12	6.25	12.5	25	50	100
			Exposure No. 1			
0	14 [s]	15 [s]	16 [s]	14 [s]	15 [s]	26 [s]
24	nd	nd	nd	nd	nd	nd
48	nd	nd	nd	d.A-7%	d.A-27%	d.A-73%, d.C-4%
72	nd	nd	nd	d.A-7%	d.A-73%	All Dead
96	nd	nd	nd	13*	d.A-7%	All Dead
			Exposure No. 2			
0	17 [s]	14 [s]	15 [s]	16 [s]	15 [s]	12 [s]
24	nd	nd	nd	nd	nd	nd
48	nd	nd	nd	nd	d.A-53%	d.A-33%, d.B-8%, C-8%
72	nd	nd	nd	nd	d.A-33%	d.A-42%, d.C-8%
96	nd	nd	nd	nd	d.A-7%	All Dead
			Exposure No. 3			
0	17 [s]	14 [s]	15 [s]	16 [s]	15 [s]	12 [s]
24	nd	nd	nd	nd	nd	nd
48	nd	nd	nd	nd	nd	nd
72	nd	nd	nd	nd	d.A-29%	d.A-34%
96	nd	nd	nd	nd	d.A-29%	All Dead

nd: no deformity recorded and all of the larvae survived.
d. A% denotes % of Type-A deformity, d. B %: denotes % of Type-B deformity, and d. C %: denotes % of Type-C deformity.
[s]: denotes number of larvae at start of exposure (0 h).
*: denotes that 13 out of 14 larvae survived.
[f]: denotes WAF prepared by using 1.0g KCO/l seawater.

IV. DISCUSSION

The present study encompassed a detailed examination of the effect of WAF of KCO exposure on fish egg hatching and the survival of larvae, to gather evidence of the types of deformities in eggs and larvae on exposure to KCO and to gain insight into the causative component(s) of KCO responsible for producing harmful effects on the early life stages of fish. In the present study, embryonated eggs and larvae of hamoor fish were used for exposure studies to KCO WAF prepared by a single oil loading and subsequent dilutions. Other conditions for WAF preparation were kept constant. The Chemical characterization of exposure solution revealed levels of BTEX, TPH and PAHs in KCO WAF which are known to exert toxic effects on the early life stages of fish. As fish can quickly absorb a part of the volatile; mostly BTEX compounds (monocyclic aromatic hydrocarbons, which consist primarily of benzene, toluene, ethylbenzene and xylene) in WAF with adverse consequences to biological organization [2], [3]. No effect of exposure to KCO WAF was observed on the percentage of hamoor egg hatching and near 100% hatching was found. Eggs that were not hatched by 48 h were not hatched later and were identified as dead eggs. No abnormalities were noticed at the egg stage, as they appeared to be more resistant to the toxic action of KCO WAF which was reflected in the successful hatching process, most probably, because of the egg envelope (chorion) which serves as a protection to the embryo from the chemical, physical and biological pressure in the environment [25]. The chorion protection ability is mainly caused by an enzyme responsible for hardening the egg envelope (chorion) in rainbow trout, *Oncorhynchus mykiss* is transglutaminase (TGase). Malformations after exposure to petroleum hydrocarbons associated with embryonic or larval fish occurred because of enzyme inhibition by pollutants, tissue injury and deformity types can range from eye deformations, disturbed vertebral column, jaw abnormalities, aberrant behavior such as impaired swimming patterns and catching behaviors, reduced length, slower growth rate and increased susceptibility [11].

Hamoor larvae which hatched during exposure showed 100% larval survival until 96 h of exposure in controls, but experienced a reduction in survival at a lower exposure concentration and none of the larvae survived by the end of exposure period at the highest exposure concentration. The components of WAF have been considered to be an important determinant of the petroleum toxicity in accidental spills [1]. In the present study 2.22 mg/l TPH caused fish larval deformity at ≤1.0g KCO/l loading which was higher that what was observed in literature [26] where hatchability was decreased at 0.5-mg TPH and above. These finding support in the fact that early life stages of fish tends to be in the most sensitive stages to crude oil exposure [27], [28]. There is no report on the visual evidence regarding the deformity types of hamoor larvae upon exposure to KCO WAF. The most common type of deformity was Type-A (lordosis-inward curvature of spine or V-shaped) followed by Types-B (kyphosis- hunchback) and Types-C (scoliosis-lateral bending of spine), which were in agreement with deformities detected in fish [29]. Craniofacial, cardiovascular, vertebral and yolk sac deformities are the most common types of body malformations reported in literature [30-35]. WAF prepared at 1-g KCO/l showed a linear response in percentage abnormality of larvae expose to dilution series.

Studies on rainbow trout (*Oncorhyncus mykiss*) larvae exposed to crude oil loadings similar to the ones used in this study resulted in developmental abnormalities in 30% of exposed larvae [36]. The most frequent type of deformity encountered in this study were the vertebral abnormalities

which amounted to more than 90% of skeletal defects recorded in various KCO treatments.

Craniofacial and lordosis deformities were another type of skeletal deformity that was recorded in this study, which can be attributed to exposure to three-ring PAHs like dibenzothiophene or phenantherne as reported in other studies. When the effects of seven nonalkylated PAH compounds containing 2-4 rings (naphthalene, fluorine, dibenzothiophene, phenanthrene, anthracene, pyrene and chrysene) on zebrafish development. Larvae treated throughout embryogenesis with naphthalene, anthracene or chrysene had grossly normal anatomic features. In contrast, larvae treated with fluorine, dibenzothiophere, or phenanthrene displayed dorsal curvature of the trunk and tail and significant growth reduction of the head. In addition, dibenzothiophene-and phenanthrene- treated embryos showed severe pericardial and yolk sac edema, while fluorine treatment resulted in mild pericardial edema. Treatment with pyrene resulted in mild pericardial edema and a less-pronounced dorsal curvature, and other distinct defects [17].

The types of deformity frequently observed in this study resembled the reported deformities due to certain PAHs that were also detected in WAF of KCO and may possibly be the causative agents as PAHs are known to exert distinct type of abnormalities as described and observed in the present exposure experiments [29]. The embryonic toxicity of PAHs in fish appeared to occur because of sensitivity to planar polycyclic aromatic compounds, high bioaccumulation and limited biotransformation, and exposure during critical developmental periods [15], [37].

Several PAHs like naphthalene, acenaphthalene, acenaphthene, fluorine, phenanthrene, fluoranthene and pyrene were detected in WAF of KCO prepared at 1-g KCO/l seawater whereas other PAHs were below detectable limits. Petroleum hydrocarbon concentration in Kuwait marine area that ranges from 1.05- to 26.6-µg/l seawater with average 2.36-µg/l seawater is much lower than LC_{50} values against sobaity larvae, but LOEC for deformity was close to occasionally found h

igher values in Kuwait's marine area [38]. The experimental study showed that the current contamination of petroleum hydrocarbon in Kuwait's marine area is not posing any acute hazard to fish egg hatching or the survival of hatched larvae. However, some caution is required, since occasionally EPA reports episodic contamination that may cause low grade deformities in developing larvae. Chronic exposure to low (sub-lethal) concentration of dissolved hydrocarbons may result in more serious effects on the early life stages of fish in Kuwait marine area.

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