TOXICITY OF KUWAIT CRUDE OIL AND DISPERSED OIL ON SELECTED MARINE FISH SPECIES OF KUWAIT

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Declaration

I hereby declare that this work is entirely my own, except where otherwise acknowledged, and that it has not been submitted previously for a degree at this, or any other university.

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Dedication

I dedicate this thesis to my late grandmother, my Father, mother and brother; who taught me the real meaning of family and dedication.

Abstract

Oil spill is a major source of pollution in Kuwait marine environment and oil dispersants are used as a method to combat oil spill but the adverse effects of either oil or dispersed oil is unknown to fish species local to Kuwait. Therefore, the toxicity of water-accommodated fraction (WAF) of Kuwait crude oil (KCO) and chemically enhanced water-accommodated fraction (CE-WAF) of KCO with three dispersants (Corexit® 9500, Corexit® 9527 and Slickgone® NS) were investigated against selected marine fish species local to Kuwait marine waters such as: sobaity-sea bream (*Sparidentex hasta*), hamoor-orange-spotted grouper (*Ephinephelus coicoides*), meid-mullet (*Liza Klunzingeri*), and shea'am-yellow-fin sea bream (*Acanthopagrus latus*).

Prior to exposure chemical characterization of KCO WAF and CE-WAFs was conducted for benzene, toluene, ethylbenzene and xylene (BTEX), polycyclic aromatic hydrocarbons (PAH), aliphatic and total petroleum hydrocarbons (TPH) compounds. Standardization experiments regarding oil loading and mixing duration revealed that 1 g KCO loading and 24 h mixing duration were the most appropriate experimental conditions to obtain a reproducible and stable WAF and CE-WAF solutions. In general, CE-WAF contained higher concentrations of TPH, PAHs and aliphatics compared to KCO WAF.

Exposure to KCO WAF and CE-WAF had no adverse effects on hatching success of embryonated eggs of sea bream and orange-spotted grouper exposed but larvae hatched during exposure exhibited a toxic response. Considering larval sensitivity, pre-hatched larvae of four marine fish species were separately exposed to KCO WAF and their sensitivities from the most sensitive to the least sensitive were: sea bream > orange-spotted grouper > yellow-fin sea bream > mullet pre-hatched larval stages. The sensitivities of pre-hatched larvae of sea bream and orange-spotted grouper to WAF and CE-WAF were of different degrees. For sea bream the LC_{50} values were around 0.120 g oil/L for both WAF and CE-WAF indicating that dispersant didn't increase oil toxicity, whereas for orange-spotted grouper CE-WAF (LC_{50} 0.010 g oil/L) was more toxic than WAF alone (LC_{50} 0.93 g/L). The data obtained in this study showed that most resistant developmental stage of fish to the toxicity of WAF and CE-WAFs was the egg stage >

larvae hatched during exposure > pre-hatched larvae. Exposure of pre-hatched larvae to KCO WAF induced developmental abnormalities in spinal curvature of larvae and the most prominent deformity types were lordosis, scoliosis and kyphosis compared to that of control larvae were no abnormalities were observed.

Relating toxicity data obtained in the present experimental study to actual petroleum hydrocarbon concentrations in Kuwait marine area, it was observed that current contamination level with petroleum hydrocarbons is far less than the LC_{50} determined in this study suggesting that there isn't any acute hazard to either fish egg hatching or larva survival.

Abbreviations

Alaska Department of Environmental Conservation (ADEC)

American Petroleum Institute (API)

Analysis of Variance (ANOVA)

Analysis of Covariance (ANCOVA)

API Gravity (°API)

Barrels (bbl)

Barrels per Day (bbl/d)

Benzene, Toluene, Ethylbenzene and Xylene (BTEX)

Control (C)

Carbon (C_n)

Central Analytical Laboratory (CAL)

Chemically Enhanced Water –Accommodated Fraction (CE-WAF)

Centimeter (cm)

Centimeter per Liter (cm/l)

Centistokes (cSt)

Centre National pour l'Exploitation des Oceans (CNEXO)

Central Nervous System (CNS)

Coefficient of Determination (R^2)

Conradson Carbon Residue (CCR)

Conservation of Clean Air and Water in Europe (CONCAWE)

The Chemical Response to Oil Spill Environmental Research Forum (CROSERF)

Department of Environment Food and Rural Affairs (DEFRA), UK

Delivery Into Vortex (DIV)

Delivery Not Into the Vortex (DNIV)

Dichloromethane (DCM)

Dispersed Enhanced Water-Accommodated Fraction (DEWAF)

Dissolved Oxygen (DO)

Dissolved Organic Carbon (DOC)

Effect Concentration (EC_{50})

Egg (E)

Electron Impact (EI)

Emission (EM)

Environment Protection Authority (EPA)

Equal to (=)

Excitation (EX)

Exxon Valdez Oil Spill (EVOS)

Experiment (Exp.)

Fourier Transform Infra Red (FTIR)

Gas Chromatography (GC)

Gas Chromatography Mass Spectrometry (GC/MS)

Gas Chromatography Flame Ionization Detector (GC/FID)

General Linear Model (GLM)

Gram per Liter (g/l)

Gram per Milliliter (g/ml)

High Performance Liquid Chromatography (HPLC)

Hour (h)

International Agency for Research on Cancer (IARC)

International Maritime Organization (IMO)

Kuwait Institute for Scientific Research (KISR)

Kuwait Crude Oil (KCO)

Larvae Hatched during Exposure (LHE)

Less than (<)

Less than or equal (≤)

Lethal Concentration which affects 50% of the population (LC_{50})

Liter (L)

Manual of Oceanographic Observations and Pollutant Analysis Methods (MOOPAM)

Mariculture and Fisheries Department (MFD)

Mass -to-Charge Ratio (m/z)

Mass Spectrometry (MS)

Meters per Second (m/s)

Microgram per milliliter (µg.ml -1)

Microgram per milliliter (µg/ml)

Micro Meter (µm)

Milliliter (ml)

Milliliter per Minute (ml/min)

Milligram per Liter (mg/l)

Millisiemens (mS/s)

Minute (min)

More than (>)

More than or equal (≥)

National research Council (NRC)

Nano Gram per Milliliter (ng/m)

Nano Meter (nm)

No Observable Effect Concentration (NOEC)

Not Recorded (NR)

Organization of Economic Co-Operation and Development (OECD)

Ocean Studies Board (OSB)

Occupational Safety and Health Administration (OSHA)

Parts per Thousand (ppt)

Percentage (%)

Petroleum Research Centre (PRC)

Potentiometric Hydrogen (pH)

Polycyclic Aromatic Hydrocarbons (PAHs)

Pre-Hatched Larvae (PHL)

Rapid Oil Containment (ROC)

Regional Organization for the Protection of Marine Environment (ROPME)

Revolution per Minute (rpm)

Salinity (‰)

Sea Water Soluble Fraction (SWSF)

Seconds (s)

Selected Ion Monitoring (SIM)

Sea Water (SW)

Signal-to-Noise (S/N)

Single Ion Monitoring (SIM)

Simple Green® (SG)

Spectrofluorophotometery (SPM)

Standard Error (SE)

Scottish Office Agriculture and Fisheries Department (SOAFD)

Temperature (°C) Degree Centigrade

Total Hydrocarbon Concentrations (THC)

Total Petroleum Hydrocarbons (TPH)

Ton (t)

Transglutaminase (TGase)

Ultraviolet A-Rays (UVA)

Ultraviolet Fluorescence (UVF)

United Nations Environment Program (UNEP)

United States Environmental Protection Agency (USEPA)

Volatile Organic Compounds (VOC)

Volume to Volume (v/v)

Water-Accommodated Fraction (WAF)

Water-Soluble Fraction (WSF)

Weight to Weight (w/w)

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PREFACE

This thesis consists of six chapters. In the Chapter one, a general introduction to the research topic is given. Chapter two, deals with in depth literature review and aims of the study. Chapter three deals with chemical characterization of the test chemicals analysis and consists of in depth literature review, materials and methods, results, discussion and conclusion. Chapter four deals with toxicity testing of prepared chemicals and exposure to local fish species and it consists of materials and methods, results, discussion and conclusion. Chapter five includes a general discussion of chapters three and four. Chapter six, the material, safety and data sheet of the three dispersants used is highlighted. A summery description of each chapter is given here.

Chapter 1

This chapter deals with introductory overview of the behavior of crude oil and dispersed oil in the marine environments and its adverse effect on aquatic organisms.

Chapter 2

This chapter highlights a comprehensive literature review of toxicity of crude oil and dispersed oil, oil dispersants history and laboratory exposure regime. The aims of the present study are given in this chapter.

Chapter 3

This chapter deals with in depth literature review of previously used methodology of water-accommodated fraction (WAF) and chemically enhanced water-accommodated fraction (CE-WAF) of Kuwait crude oil preparation. Factors which affect the overall consistency of the test medium are thoroughly discussed. Several experiments were conducted to characterize Kuwait crude oil WAF and CE-WAF solutions. Sections such as: materials and methods, results, discussion, are included in this chapter and conclusion is drawn in view of the obtained results.

Chapter 4

This chapter describes the previous exposure methods used in toxicity testing, and it focuses on acute toxicity testing of crude oil and dispersed oil on selected

test species local to Kuwait, LC50 determination, statistical analysis and research constraints. In addition, it includes a result section which highlights all various experiments conducted with four fish species after exposure to Kuwait crude oil WAF and CE-WAF solutions. A thorough discussion of the obtained results is included in this chapter and a conclusion in drawn in view of the results.

Chapter 5

This chapter presents a general discussion of chemical characterization of Kuwait crude oil WAF and CE-WAF, their acute toxicity on selected native marine fish species f Kuwait, and conclusion.

Chapter 6

This chapter includes appendices for the safety, material and data sheet of the three oil dispersants used in the study.

Chapter One

INTRODUCTION

1. 1 Introduction

In the 21st century the Arabian Gulf became increasingly one of the busiest regions in the world for shipping; receiving globally the highest number of merchant ships and oil tankers to load crude oil and other goods. Since the Arabian Gulf countries hold approximately two-thirds of the world's crude oil reserves, it produces about 25% of the world's oil; and 11% of that estimate (1.7 million bbl/d) is accounted for the State of Kuwait. In 2002 only, the gross oil imports of the Arabian Gulf countries averaged 10.6 million barrels per day (bbl/d) and this estimate account for 27% of the total gross oil imports for the Organization of Economic Co-Operation and Development (OECD) (www.eia.doe.gov, 2001) (Figure 1-1).

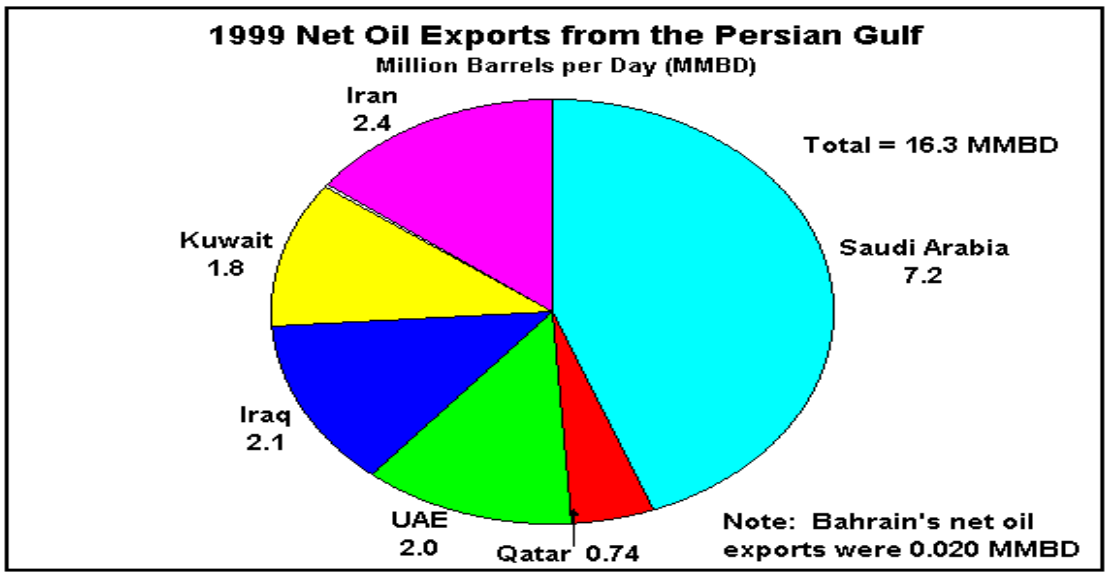


Figure 1-1: 1999 Net oil exports from the Persian Gulf (from www.eia.doe.gov, 2000)

As long as crude oils, chemical substances and other related petroleum products are transported across global regions by ships or pipelines there will a possibility of an oil spillage with potential risk to cause environmental damage (Daling et al., 1990). When transporting crude oil from the Arabian Gulf region, oil spill accidents are common phenomena and the release of hazardous substances are likely to eventually create substantial environmental damage and have serious consequences for the marine

ecosystem. A spillage of approximately 182, 900 metric tons of oil per year into the Arabian Gulf at the mid-1976 export rate was caused by tankers which carry crude oil. A total of approximately 25,000 metric tons of crude oil is spilled per year into the Arabian Gulf from various sources such as pipelines and off-shore drilling operations. Moreover, an additional 16, 800 metric tons of crude oil enters the Arabian Gulf from coastal refineries, natural seeps, and municipal and non-refining industrial wastes (Hayes et al., 1977). There are numerous reasons for some of the large scale oil spills that occur in the world's oceans and most importantly about 75% of these oil spills were caused by ship collisions and groundings while approximately 8% were caused by oil transfer operations (Figure 1-2).

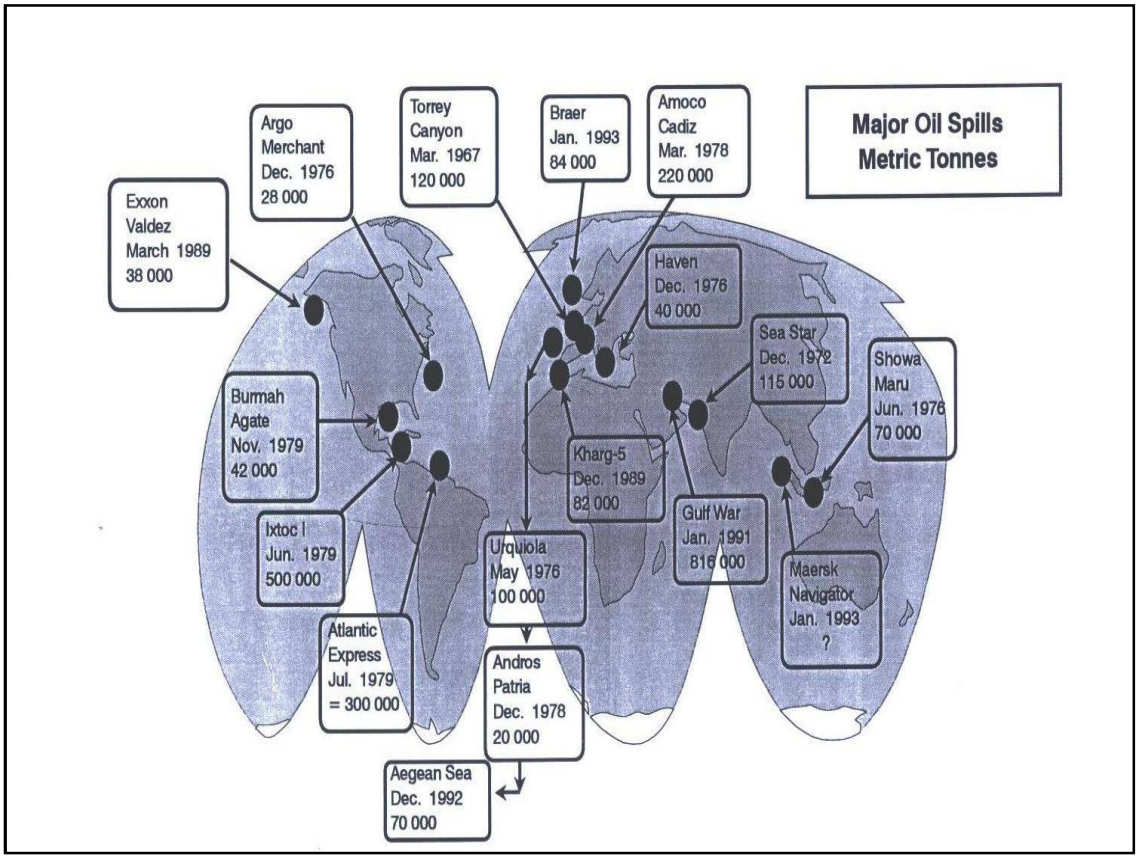


Figure 1-2: Major oil spills accidents in the world that occurred from 1967 to 1993 (from Pearce, 1993)

In 1991, the Arabian Gulf experienced an oil spill accident which was estimated to be around 816,000 metric tonnes (t) in volume (Pearce, 1993; SOAFD, 1993; Wolf et al., 1993). It is estimated that the Arabian Gulf region receives approximately around

1.2 million barrels of crude oil which are yearly spilled by accident (GEO, 2000; Al-Majed et al., 2000). And in a global scale, it's been estimated that 1.3 million metric tons of petroleum pollutants may be discharged into the world's oceans on an annual basis and the main sources of such pollutants can originate from both anthropogenic and natural inputs (NRC, 2003). In the coastal areas of the Arabian Gulf there are 25 major oil terminals which are continuously utilized to load crude oil. Since the geological nature of Arabian Gulf is considered to be semi-closed in nature, the reliance of the Gulf States on this water body for navigation, drinking-water production and seafood have further exacerbated the risks of marine pollution and environmental stress; this applies equally to the State of Kuwait as to other Gulf States. Kuwait is considered to be one of the major oil-producing countries in the world and in this century, marine pollution became one of the most significant environmental issues for Kuwait with oil input from waste discharged estimated to be 26,905 t/year (UNEP, 1999). Petroleum pollutants can be accumulated in the marine environment, such as along shorelines with the assistance of various environmental parameters such as the wind, sea currents, and the transport mechanisms. When the coastline becomes the destination of the traveling crude oil pollutants, shoreline dwelling organisms in turn become subjected to toxic contaminants exposures (Mueller et al., 1999) and (Mueller et al., 2003). Oil spills in the marine environment pose a severe threat to aquatic organisms such as: fish, bivalves, crustaceans, algae and other ecologically and commercially valuable marine resources (CNEXO, 1981; Kocan et al., 1996; Marty et al., 1997; Lancaster et al., 1998). The concentration of spilled oil in the shallow sub-tidal and inter-tidal areas will have long-term damaging effects on the biological productivity and efficacy of the Arabian Gulf in general; and on the State of Kuwait in particular. Since these distinct regions are considered to be fundamental feeding and breeding grounds for diverse aquatic and terrestrial species (Hayes et al., 1977).

1. 2 Marine Pollutants

Pollutants are defined as substances which exist in quantities that induce undesirable effects and are introduced into the natural environment as results of the various activities performed by humans (Elliott, 2003). In the 21st century, global shipping became one of the most prominent and essential methods of transportations in the world. It moves approximately 80% of the world's supplies and it's significant to

world trade (NRC, 1996). As well as the many advantages of shipping worldwide like the transportation of commodities such as: crude oil, merchant goods, international travel, etc., there are many disadvantages. Accidental oil spills, the release of organic waste originating from cattle and humans in merchant ships and the introduction of aquatic invasive species via transport ship ballast water (Tamburri et al., 2002).

1. 3 Crude Oil and Oil Spills

Key sources of oil spillage at sea need to be considered and all fall under the umbrella of marine transportations. Oil tankers, lighters, barges and off-shore operations for the exploration and production of petroleum hydrocarbons can be effective sources which can introduce oil to marine environment (NRC, 1989).

The amounts of oil involved in a spill is a key element in determining the extent of the ecological damage to the aquatic habitats specifically the coastal regions that are in close contact with the spilled oil. There are many significant factors which govern the severity of an oil spill and the consequent fate of oil pollution. Those factors are: in situ water quality parameters like (water depth, temperature and salinity), chemical composition of oil and seasonal effects. The physical nature of marine habitat which will be in contact with the dispersing oil droplets is also an essential factor that needs to be considered. In addition, physical sea water factors such as: prevailing wind speed, current direction and wave energy can be of a relative importance. Moreover, the efficiency of oil spill contingency plan which involves the type and speed of the clean-up process of an oil spill (Dipper, 1991).

Natural processes effectively act on crude oil products which have been spilled offshore and ultimately these processes degrade and disperse these oil compounds. These processes include the following: evaporation, dissolution, dispersion, emulsification, sinking, photooxidation, and finally biodegradation. Among these processes, that exerts the most prominent effect on the action of dispersion is emulsification. Emulsification can be defined as the incorporation of water droplets into oil (Figure 1-3).

Another factor that should be considered when combating an oil spillage is the viscosity of oil. Viscosity of oil tends to be enhanced rapidly and dramatically with introduction of weathering processes. Then, the more viscous oil tends to be the more difficult it can be dispersed and eliminated; which is why an immediate response should

be applied within the first few hours to combat the spillage. With time, oil slick areas will have the tendency to spread out in a linear fashion and the area affected will be increased by the action of current shear even more rapidly (NRC, 1989).

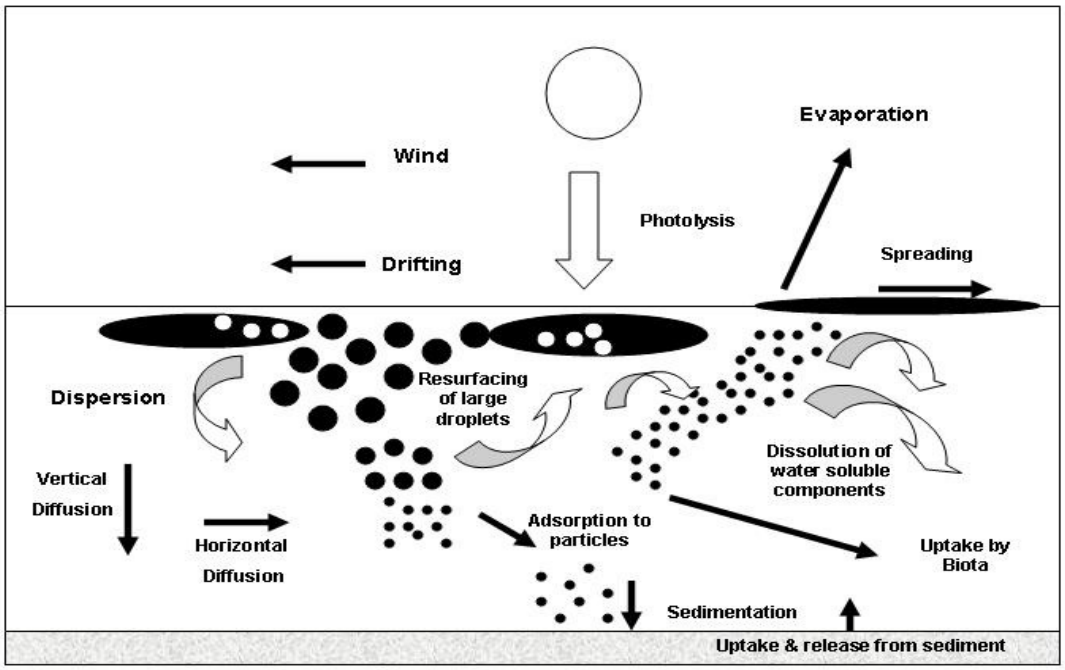


Figure 1-3: The process of oil dissolution in the marine environment in the case of oil spill incident (from Daling et al., 1990)

1. 4 Crude Oil

Crude oil, which gives rise to most oil spills, is composed of a mixture of a hundreds of different compounds. Crude oils are considered to be naturally occurring substances which are derived from the decomposition processes of organic matter such as dead animals and plants with the aid of environmental factors like pressure and high temperature over thousands of years. In many areas, crude oil has migrated considerable distances and accumulated in porous rock formations. Those formations were overlaid by impermeable rock formations which acted as a barrier (petroleum trap) to prevent further movement of crude oil. Crude oils consist of a complex mixture of several compounds which serve as the 'backbone' of any crude oil type in the world. Such compounds can be a combination of hydrocarbons like: parrafins (30%, range 15-60%), naphthalenes (19%, range 30-60%), aromatics (15%, range 3-30%) and asphalitics (6%)

(Hyne, 2001). Also, which consists of straight and branched chain alkanes aromatic cycloalkanes, aliphatic and alicyclic hydrocarbons covering the carbon range from C1 to C60. In addition, crude oil consists of other compounds like: oxygen, sulfur and nitrogen and dissolved gases like hydrogen sulfur (CONCAWE, 2001) and various metals such as: nickel, vanadium, iron, zinc, and copper (Tissot and Welte, 1984).

Crude oil types differ widely in terms of appearance, ranging from thin light crude oil (which consists primarily of gasoline-quality stock) to heavy thick materials which resemble tar in appearance (Table 1-1). Depending on the oil producing region, the chemical composition of crude oil can vary enormously, even from within a particular geological formation. More than 98% of the compounds present in petroleum oil are hydrocarbons (NRC, 1985). An average crude oil consists of 0.1% salts and minerals, 1.0% oxygen, 1.0% nitrogen, 1-3% sulfur, 14%hydrogen, and 84% carbon. Crude oils have a complex composition with unique chemical and physical properties which vary from one type to another. Crude oils from different oil producing regions possess different compositional properties such as: partition coefficient, melting point, water solubility characteristics, vapor pressure and boiling point. On the other hand, despite those variations in crude oil characteristics between the various oil producing regions, there are some general characteristics relating to the environmental behavior of crude oil according to surrounding environmental conditions (Head et al., 2003). As an example of crude oil type from a certain oil producing region such as Kuwait, where it is considered as one of the major oil exporting countries in the world, the crude oil which is exported (called Kuwait crude oil, export) is a mixture of several oils which are produced from various oil fields. The mixed oil composition depends on several factors such as: 1) refining process requirements, 2) availability of the different crude oils, and 3) consumer specification and other related economical factors (Saeed et al., 2000).

The chemical compositional changes which occur to crude oil when there is an oil spillage at sea have been comprehensively studied and reviewed (see for example, Dean, 1968; Smith, 1972; Nounou, 1980; Robotham and Gill, 1989). Instantly after the release of crude oil into the sea, a series of environmental factors (both biotic and abiotic) start to affect the physical and chemical composition of the crude oil. Partitioning of crude oil components into several environmental compartments will occur. Three distinctive environmental compartments will be generated which are: 1) Lower molecular weight components, 2) Intermediate fractions, and 3) viscous and heavy components. The lower molecular weight fractions may become volatile in the atmosphere or dissolve in the sea water and disappear rapidly within 2 days; while the

second type, the intermediate components, may spread out and float on the sea where they may become adsorbed to marine sediments or they may form emulsions. Finally, the third type which is the heavy viscous components may agglomerate and solidify and either float or sink in the sea water which ultimately can be adsorbed to the sediments or soil. They also can remain in the water column for longer periods such as up to 9 days (Yamada et al., 2003). What classify crude oil as light or heavy crude oil is its heavy crude oil because its density and specific gravity is higher than that of light crude oil. In other words, light crude have specific gravity (> 31.1 °API), medium crude oil (between 22.3 °API and 31.1 °API), and heavy crude oil (< 10.0 °API)(www.dnr.louisinana.gov, 1989; Desseault, 2001).

Table 1-1: Several crude oil compositions

Crude Source	Parrafins % Volume	Naphthelenes % Volume	Aromatics % Volume	Sulfur % Volume	API Gravity (°API)
Light Crude Oils					
Saudi Light	63	18	19	2.0	34
South Louisiana	79	45	19	0.0	35
Beryl	47	34	19	0.4	37
North Sea Brent	50	34	16	0.4	37
Nigerian Light	-	-	-	-	-
Lost Hills Light	Non-Aromatics 50%		50	0.9	-
USA Mid Continent Sweet	-	-	-	0.4	40
Mid Range Crude Oils					
Venezuela Light	52	34	14	1.5	30
Kuwait	63	20	24	2.4	31
USA West Texas Sour	46	32	22	1.9	32
Heavy Crude Oils					
Prudhoe Bay	27	36	28	0.9	28
Saudi Heavy	60	20	15	2.1	28
Venezuela Heavy	35	53	12	2.3	24
Belridge Heavy	Non-Aromatics 37%		63	1.1	-

(From IARC, 1989; Mobil, 1997; OSHA, 1993)

In the Arabian Gulf several factors affects the dispersal of crude oil on the sea such as: 1) tidal currents, 2) general Arabian Gulf circulation patterns, and 3) the wind in which oil moves in the direction of the wind at 4% of the wind velocity (Hayes et al., 1977). Immediately after oil spillage, evaporation processes reduce the volatile components of crude oil at a rate which is considered to be proportional to their vapor pressure. The duration of the evaporation process may be as much as several weeks, leading eventually to the removal of between 30-50% the total hydrocarbons (Clark and Macleod, 1977). Ultimately, the released oil is therefore categorized into three distinctive phases 1) Vapor phase, 2) dispersed and dissolved phase, and 3) bulk oil phase. The phase which constitutes a degree of significance is the dissolved phase in which its compositions is severely altered from that of the original oil since its individual compounds become soluble in sea water when the oil is spilled. Additional

photochemical oxidation and microbial degradation will induce further alteration to crude oil composition in which eventually these natural mechanisms will increase the toxic effects on the marine ecosystem (Tilseth et al., 1984; Sydnes and Burkow, 1985; Ehrhardt et al., 1992).

1. 5 Toxicity of Crude Oil

Spilled oil in the sea water surface has several significant consequences. For instance, it limits the exchange of gases between the water body and atmosphere, it covers fish gills and renders breathing impossible and it affects surface dwelling aquatic organisms by entrapping them in the thick and viscous oil medium which eventually kills them (Wells et al., 1995; Spies et al., 1996). Spilled oil also poses certain threats to microscopic organisms in the marine ecosystem like phytoplankton by directly decreasing its photosynthesis rate and depressing growth and respiration rates. In the case of zooplankton and early-life stages of other marine organisms, it causes developmental abnormalities and mortality (Afolabi et al., 1985; NRC, 1985; Otitoloju and Adeoye, 2003; Powell et al., 1985).

One of the major constituents of crude oil is polycyclic aromatic hydrocarbons (PAHs) and these are regarded as a chief determinant of oil toxicity to marine organisms. As has been determined, the individual constituents of petroleum certainly exhibit different levels of toxicity. PAHs are considered to be carcinogenic, neurotoxic and mutagenic (Saeed et al., 2000). Moreover, there are biological reactions toward the introduced toxicants which include: hemorrhages, edema, developmental retardation, and spinal deformation. Crude oil contains low-molecular weight and high-molecular weight hydrocarbons and there is a distinctive toxicity variation between the two categories. Low-molecular weight hydrocarbons which possess one or two benzene rings are considered to be relatively soluble in water with octanol/water partition coefficient ($\log K_{ow}$) < 5 and they tend to rapidly reach high concentrations in the water medium in a short period of time estimated to be around a few hours subsequent to an oil spill. Nevertheless, since they are very vulnerable to evaporation process, they are not considered to be highly persistent in the marine environment. They are thought to be a major environmental concern due to the fact that they are able to break through cell membranes to induce a toxic effect (like narcosis, for example). Narcosis determination in an organism's cell can be considered as a toxicity approach resulting from the

presence of foreign molecules (xenobiotic) in hydrophobic or lipid tissues that ultimately act as a depressor and disruptor of various cellular functions (Franks and Lieb, 1978; Abernathy et al., 1988). And it can manifest itself in multiple ways like: mortality, immobility and loss of equilibrium (Rogerson et al., 1983; Mackay and Hughes, 1984; Bobra et al., 1985; McCarty et al., 1992, Thornburg, 2004).

The second category of larger PAHs is the high-molecular weight hydrocarbons which possesses three to five benzene rings. They are considered to persistent in the marine environment and less soluble in the water column with greater toxicity effects than the low-molecular weight hydrocarbons. As low-molecular weight hydrocarbons (log K_{ow} < 5.5) with high solubility partition into the aqueous phase, they will rapidly bioaccumulate across gut epithelium or external permeable membranes when water is ingested. However, in the case of high-molecular weight hydrocarbons (log K_{ow} > 5.5) with low solubility, fewer hydrocarbons will partition into the aqueous phase (BATTELLE, 2007).

Genotoxicity and oxidative stress are some of the toxic manifestations of high-molecular weight hydrocarbons (Marvin et al., 2009). Equally, low and high-molecular weight hydrocarbon concentrations will be enhanced in the water column after the application of dispersant to combat an oil spillage on the sea, hence, their toxicities especially the high-molecular weight hydrocarbons will be elevated as it will be demonstrated later (Couillard et al., 2005).

1. 6 Oil Spill Response Strategy

Various methods of combating oil spills have been the subject of worldwide research since the 1960's. Various control strategies have been developed as methods of combating spillage of crude oil in the marine environment and those means involving recovery techniques such as: skimmers, pumps, burning, booms, or dispersants or involving containment procedures (Westermeyer, 1991). But, each method has certain limitations regarding its application in the containment of oil in the marine environment. For instance, mechanical recovery is limited by the action of sea currents, wind and other sea conditions, therefore; this method only allow for the recovery of a small fraction of the spilled oil. Booms are another method of oil spill containment in which an oil spill is isolated and surrounded by a boom to block the passage of oil slick to sensitive marine areas such as fish farms, etc. However, there are limitations for such

method, in which the boom will not contain oil above the maximum speed of 1 knot (0.5 m/s). If the current speed is higher than 1 knot, the boom will not be able to contain the spill and the oil will escape from under the boom. Skimmers are suction devices designed to recover oil from the surface of the water contained within the boom and the oil can be sucked into a storage tank in a close vessel. Oil viscosity, amount of storage and rate of recovery are considered as limitations for such method. ROC (rapid oil containment) BarrierTM is another method which uses high-extension sorbent barrier for the containment of an oil spill. Therefore, the effectiveness of mechanical recovery technique for an oil spill is dependent on spill situation itself. A more common method of combating oil spill is the application of oil spill dispersants for the rapid removal of spilled oil from the surface of the sea and transfers it to the water column where it can be diluted and then biodegraded (ROPME, 1998; www.murrenhil.com, 2008).

1. 7 Oil Dispersants

Due to the growing public concern regarding the consequent effects of oil spills (both acute and chronic effects) on the aquatic ecosystem and whether these affect aquatic plants and organisms, in pelagic and benthic systems and in on-shore and off-shore habitats; several means of oil spill combating techniques have been developed in the past fifty years. Chemical dispersants have gained a broad approval as a potentially effective and beneficial technique in the oil spill response strategy evaluation of their toxicity against aquatic organisms. The Regional Organization for the Protection of Marine Environment (ROPME) have prepared a promising program for the oil spill response in the case of an accidental release of oil in the Arabian Gulf to minimize the threat posed by an oil spill to aquatic plants and animals and more generally to marine resources (CNEXO, 1981; Kocan et al., 1996; Marty et al., 1997). Dispersants can be defined as chemical formulations which consist of individual components called surfactants. Surfactants are a specific chemical compounds which posses two distinctive groups called oleophilic (oil liking) and hydrophilic (water liking) groups. The primary function of these chemicals is to reduce the interfacial tension between oil and water and assist in the formation of minute droplets or (mixed oil surfactant micelles) which move and disperse in the water column. This movement will further facilitate natural biodegradation and dispersion. Finally, the size of the small oil droplets will be decreased; in addition oil droplets will be dispersed in the water column which will lead

in turn to an increase of the surface area exposed to water and it will further facilitate and enhance the biodegradation of oil (ROPME, 1998).

Dispersants are defined by Couillard et al., (2005) as chemicals which when applied result in oil being diluted in the sea water to levels which are considered to be nontoxic to marine life. The dispersed oil will increase the degradation rate through weathering processes. There are three essential component groups in which oil spill dispersants are composed of: 1) solvents (like water and hydrocarbons), 2) surface-active agents, and 3) stabilizing agents. By dispersing oil slick, marine biota such as plants, mammals and birds will be protected from contamination and the severe effects of crude oil toxicity when it comes to contact with the floating oil (www.amsa.gov.au, 2006; Canevari, 1973, 1978; NRC, 1989). There are safety concerns which should be regarded when applying oil dispersants like the possibility of explosion hazards in the case of volatile crude oil spill. Furthermore, the application of dispersants may potentially enhance the risk of fire incidents, as they tend to break up oil they increase the amount of total hydrocarbons in the atmosphere. Accordingly, as a safety measures it is recommended that dispersants shouldn't be applied in the vicinity of a populated area or near drinking water supplies and more importantly they should not be used in freshwater environments (Pollino and Holdway, 2002 a, b).

1. 8 Dispersion Mechanism

There are certain factors which govern the ability of oil to disperse in sea water and the absence of such factors, dispersion of crude oil is unlikely to occur. Firstly, as mentioned previously, chemical dispersants induce the reduction of surface tension between sea water and crude oil if only one essential rule is followed and that is the proper application of dispersant as a mist not poured or sprayed onto the oil slick. Since some crude oils are considered to be less dispersible than others because of the formation of water-in-oil emulsions and enhanced viscosity. Viscosity of the spilled oil is enhanced at the time of weathering and at the presence of lower temperature. Secondly, physical parameters like wave energy play an integral role in dispersion process in terms of achieving optimum crude oil dispersion. Wave energy will break the oil into small droplets which will be mixed in the upper water column as a "cloud" of minute and buoyant globules. Consequently, this cloud of droplets will be further mixed deeper in the water column via current shear and Langmuir circulation in a lateral

fashion. Other natural mixing process such as water currents and tides will transport the diluted material in progress out of the oil spill zone. Thirdly, dispersed oil's nature does not allow it to sink in the water column and the maximum sea depth at which deliberately dispersed oil has been measured is one order of 10 meters. Conversely, in shallow waters with depths ≤ 10 meters dispersed oil will certainly be in contact with bottom sediments which will in due course affect benthic organisms. Fourthly, time is a significant factor which plays a role in decreasing oil dispersion due to the continuous and prevailing weathering processes at sea. Fifth, the actual dosage rate of dispersant applied which entirely depend on the application technique followed and mechanical equipment utilized. Furthermore, hydrocarbons, aspahltenes and natural surfactant concentrations play a significant role in oil dispersion. Finally, the new generation of oil dispersants causes less toxicity to marine organisms than the ones previously used in the 1960s and they are less toxic than the crude oils being dispersed. The short-term toxicity of dispersed oil will decline with decreasing concentrations in the dispersing plumes (Mearns, 1999; NRC, 1989).

1. 9 Oil Dispersants History

The term "dispersant" gained a wide acceptance in oil response strategy following the devastating *Torrey Canyon* Spill of the English coast in 1967. Approximately 1-million barrels (bbl) were spilled in the sea and as a response strategy, 10,000 bbl of several chemical substances which consisted primarily of degreasing solvents were sprayed on the waters and along the coastline. The earliest dispersant which were applied in the *Torrey Canyon* spill weren't actually intended to disperse spills, as a result, they showed a toxic behavior on aquatic plants similar to the toxic behavior of crude oil being spilled on the sea (NRC, 1989; Smith, 1968). The National Research Council (NRC) in 1989 extensively documented in its report the history of dispersant use and research during the 1980s period. More importantly since the *Torrey Canyon* disaster, the NRC have accumulated over 30 years of experience involving laboratorial testing, ocean modeling and simulation research, experimental oil spill and fate of dispersed oil, and acute and chronic toxicity of oil dispersants (Mearns, 1999).

Crude oils are subjected to weathering processes when spilled on the sea and this situation limit the application of chemical dispersants. Freshly spilled crude oils will have the tendency to become stickier the longer the period they are exposed to sea

water. Time is an essential and critical element in dispersant application strategy since the response time should be within certain frames which are between six and twelve hours in the affected area and decision should be made instantly to whether or not to use dispersants. Crude oil type certainly influences the effectiveness of dispersant application since it has a major impact on oils which easily flow rather than the thicker oils which have slower flow rates like for example Bunker C. oil. Those limiting factors tend to encourage the development of regional oil spill response competence throughout the Arabian Gulf countries since they are an integral part of the ROPME sea area (ROPME, 1998). ROPME have performed an extensive work on the oil spill response strategy and the role of dispersant applications in the Arabian Gulf and it had provided a provisional approval to a list of chemical dispersants indicated in Table 1-2 based on their previous acceptance in the list of approved chemical dispersants in either of the following three countries such as: United Kingdom, United States of America and France. Despite the approval of these chemical dispersants, their respective toxicity is not known against native aquatic organisms of the ROPME sea area. Accordingly, it is imperative for Kuwait and other Arabian Gulf states to develop a regional capability for toxicity testing of chemical dispersants against native marine species (ROPME, 1989).

Table 1-2: List of oil spill dispersants provisionally approved to be used in the ROPME area

No.	Dispersant Name *	Manufacturer
1	Corexit® 9500	Nalco/Exxon Chemicals, USA
2	Corexit® 9527	Nalco/Exxon Chemicals, USA
3	Dasic Slickgone® NS	Dasic International, UK
4	Dispolene® 36S	SEPPIC, Paris, France
5	Dispolene® 38S	SEPPIC, Paris, France
6	Finasol® OSR-52	Fina Chemicals
7	Gamlen® OD 4000(PE998)	Gamlen Industries, France
8	Inipol® IP 80	CECA SA, Paris, France
9	Inipol® IP 90	CECA SA, Paris, France
10	Inipol® IPC	CECA SA, Paris, France

*** Dispersants intended to be sprayed near from ships or water being diluted from ship or aircraft (from ROPME, 1989).**

1. 10 Approval of Dispersants

In order for a new chemical dispersant product to be registered and approved for licensing or registration by the regulatory authorities and agencies like (ROPME), it has to undergo toxicity testing against representative marine species under standardized and controlled laboratorial conditions. In addition, authority coordinators can compare the toxicity of various dispersant products which will enable them to authorize the application of such new product. The generated toxicity data will serve as a basis for investigation when the toxicity of dispersed oil needs to be examined (Singer et al., 1996; NRC, 1989). Toxicity of chemical dispersants alone is considered to be insignificant source to indicate the total harm such chemicals induce, since dispersants are applied on oil and oil is known from documented studies to cause higher toxicities than dispersants. Thus, it is more efficient to evaluate the toxicity of oil mixed with dispersant to duplicate the natural mixing phenomena of dispersant with sea water.

1. 11 Toxicity Testing

Toxicity is defined by (Rand and Petrocelli, 1985) as the capacity of a chemical substance to cause an adverse effect on the living organism. Toxicity can be quantified as an effect concentration (EC_{50}) or lethal concentration (LC_{50}) at a specific time. In addition, concentration unit can be expressed as gram per liter (g/L). Toxicity tests are normally conducted to predict or assess the effect of certain chemical substance such as: crude oil, industrial waste discharged, pesticides, PAHs and etc, on selected test organisms (Di Giulio and Hinton, 2008). Also, toxicity tests are applied to determine the relative toxicity of such chemicals against the exposed biota and the mechanism by which toxicity occurs. There are numerous methods to measure toxicity depending on the objective of the study, the duration of the test and the toxic effect examined; and they can encompass several trophic levels of organisms. Standardized toxicity tests have been developed over the years for the following effects: 1) acute toxicity, 2) subchronic toxicity, 3) chronic toxicity, 4) genotoxicity, 5) carcinogencicty, 6) dermal toxicity, 7) neurotoxicity, and 8) developmental toxicity (Monosson, 2007).

Biological tests (bioassays) are used to determine and compare the relative sensitivities of various biological species covering ecological hierarchies and it is aimed at producing a rank order of the substance toxicities (Pace et al., 1995; Weetma`n et al., 1995).

CHAPTER Two

SCOPE OF THE STUDY

2.1 Literature Review of Toxicity Tests

A considerable volume of literature has been generated on the subject of toxicity of oil, dispersant and oil mixed with dispersant (dispersed oil). Yet, a large amount of these data fail to be compared with other toxicological findings due to the fact that there are different analytical methods followed when performing a toxicological evaluation. In addition, there is a need of more reliable analytical verifications of exposure systems so that toxicological data can be more comparable between different regions in the world (Singer et al., 2000).

The rational for normally selecting different species that represents different trophic levels in the marine environment is that each species represents: different life spans, distinctive developmental stages, different lipid contents for energy storage, and diverse susceptibility to marine contaminants (Chapman and Riddle, 2005; Hansen et al., 2011).

Among the numerous examples in literature related to toxicity testing of crude oil, oil dispersant and dispersed oil are the early researches done by Singer et al., (1993) in which they examined the effects of chemical dispersants Slik-A-Way® and Nokomis® on two marine species, the crustacean kelp forest mysid (*Holmesimysis costata*) and the mollusk, the red abalone (*Haliotis rufescens*) using an acute, flow-through, spiked-exposure test. The two surfactants consisted of a complex mixture of anionic and nonionic solvents and surfactants and the first type of dispersant (Slik-A-Way®) exhibited more toxicity to both species than the second one Nokomis®. The differences in the two toxicity behaviors of the two dispersants may be due to differences in surfactant formulations. The median-effect concentrations for Slik-A-Way® dispersant ranged from 0.017-0.024 initial (g/L) for *Haliotis rufescens* and 0.026-0.035 initial (g/L) for *Holmesimysis costata*, whereas Nokomis® 3 median-effect concentrations ranged from 0.021-0.024 initial (g/L) for *Haliotis rufescens* and from 0.118-0.123 initial (g/L) for *Holmesimysis costata* .

Later, Singer et al., (1995) determined the acute toxicity of different types of surfactant-based oil dispersant, Corexit® 9554 using closed flow-through system was used as an exposure regime against various marine species such as: kelp forest mysid

(*Holmesimysis costata*), a macroalga, (the giant kelp (*Macrocystis pyrifera*), a mollusk, the red abalone (*Haliotis rufescens*), and topsmelt fish (*Atherinops affinis*). The four species exhibited various degrees of sensitivities to Corexit® 9554 dispersant in which the red abalone mollusk *Haliotis* was more sensitive followed by the giant kelp *Macrocystis*, then the topsmelt fish *Atherinops sp.*, followed by the kelp forest mysid *Holmesimysis costata* as the least sensitive. Estimates of the median lethal concentration for the four species examined ranged from 0.008 – 0.184 initial (g/L). It was inferred that response differences between the four species was related to the morphological and physiological variations.

Singer et al., (1996) again investigated the acute toxicity effect of new Corexit® dispersant series, Corexit® 9500, on the early life stages of the red abalone (*Haliotis rufescens*) and the kelp forest mysid (*Holmesimysis costata*). A spiked-concentration test was conducted with closed flow-through system using UV spectrophotometer to measure dispersant concentrations in real time. The median-effect concentration ranged from 0.0128 – 0.02 g/L for (*Haliotis rufescens*) and for (*Holmesimysis costata*) the range was from 0.158 – 0.245 g/L. A pattern emerged after conducting these two tests in terms of their sensitivities to the new dispersant series and it was concluded that the new Corexit® 9500 exhibited similar toxicities to Corexit® 9527 and 9554. Corexit® 9500 was manufactured to be used on higher viscosity oils and emulsions and it is a reformulation and an extension of Corexit® 9527.

In 1998, Singer et al. further investigated the acute effects of untreated and dispersant-treated Prudhoe Bay crude oil on the early life-stages of three distinctive marine organisms (mysid, red abalone and topsmelt). The investigation involved the identification of which water-accommodated fraction of dispersed and undispersed crude oils was relatively more toxic and it was determined that it was dependant on: test species examined, time period, and test endpoint. The collected data demonstrated that at roughly equivalent hydrocarbons concentrations, untreated crude oils resulted in higher initial concentrations (< 1 h) in topsmelt and mysid tests, while the dispersed crude oil solutions exhibited an elevated levels of larval abnormality in red abalone test and high mortality levels in mysid tests. It was concluded that among toxicity tests which showed higher sensitivities to untreated oil solutions was the topsmelt, while the mysid was the most sensitive species to dispersed oil solutions.

Corexit® 9527 toxicity was further investigated by George-Ares et al., (1999) in which they compared two test methods for the characterization of Corexit® 9527 and four additional dispersant formulations on mysid (*Mysidopsis bahia*). Two rapid

screening tests were used, Microtox and Mysid IQ Toxicity Test. The toxicity test included a 96 h exposure and survival of the mysid was recorded at 3, 6, 9, and 24 h. Since the test was a short-term one, it was consistent with field exposure times and fairly accurate to exposure periods used in Microtox and Mysid IQ Toxicity Test. The mortality of mysids was recorded and it was ≤ 5% for all test material at nominal concentrations 0.0063 and 0.125 g/L and exposure periods from 3 to 24 h. It was concluded that the early mortality observations during the 96-h exposure period didn't indicate an adequate foundation for the purpose of comparing rapid screening test results.

Another group of workers Mitchell et al., (2000) examined the acute and chronic toxicity effects of other dispersant products, Corexit® 9527 and Corexit® 9500, water accommodated fraction (WAF) of crude oil and dispersant enhanced WAF (DEWAF) against green hydra (*Hydra veridissima*). The mean (SE) 96 h LC_{50} values for Corexit® 9527 and Corexit® 9500 were 0.23 (0.0048) g/L and 0.16 (0.0023) g/L. The mean (SE) 96 h LC_{50} values for the WAF of crude oil (Bass Strait), Corexit® 9527 DEWAF and Corexit®9500 DEWAF were 0.0007 (0.0001) g/L total petroleum hydrocarbons (TPH), 0.009 (0.0005) and 0.0072 (0.1) g/L.

Chemical dispersants such as Corexit® 9527 was further tested by Barron et al., (2003) in which the photoenhanced toxicity of weathered Alaska North Slope crude oil (ANS) was examined on the eggs and larvae of Pacific herring (*Clupea pallasi*) with and without the addition of Corexit®9527 dispersant. ANS crude oil exhibited more acute toxicity to fish larvae at aqueous concentrations < 50 µg /l (TPAH) and both the median lethal (LC_{50}) and effective concentration (EC_{50}) declined with time after oil exposure. Sunlight has also increased the toxicity of crude oil after exposure (2.5 h/day for 48 h). The toxicity of chemically dispersed oil was similar to oil alone in the control and ultraviolet radiation treatments (UVA), however oil + Corexit® 9527 was notably more toxic in the sunlight treatments. The resultant toxicity of chemical dispersants was due to the fact that the dispersant appeared to accelerate the polycyclic aromatic hydrocarbons dissolution into the aqueous phase. When herring eggs were exposed to the crude oil, the consequence was a yolk sac edema and a conclusion was made for this study that weathered ANS crude oil is phototoxic and that UV treatments can cause herring larvae's mortality if exposed to oil and chemically dispersed oil.

Fuller et al., (2004) have compared the toxicity of dispersant 9500®, weathered and unweathered Arabian Medium crude oil plus dispersant on two fish species (*Cyprinodon variegates*) and (*Menidia beryllina*), a shrimp (*Americamysis bahia*)-

formerly (*Mysidopsis bahia*) and the microbial test using luminescent bacteria (*Vibrio fisheri*). It has been demonstrated that the oil media prepared with dispersant Corexit® 9500 was equal to or less toxic than the oil alone test medium. In addition, continuous exposure to the test media exhibited more toxicity than the declining exposures. Unweathered oil medium which was dominated by soluble fractions of the hydrocarbons were more toxic than weathered crude oil which consisted of colloidal oil fractions. Colloidal oil fractions dominated the total concentrations of petroleum hydrocarbons in oil with dispersant media prepared with weathered and unweathered crude oil showed no considerable difference in terms of its toxicity and the toxicity was a function of the soluble crude oil constituents and not the colloidal oil.

Couillard et al., (2005) examined the effect of the dispersant Corexit® 9500 on the composition of the WAF of Weathered Mesa Light Crude oil and its toxicity on larval mummichog fish (*Fundulus heterocelitus)*. The fish species were exposed in a static renewal test to the crude oil DWAF and WAF for 96 h to investigate the changes induced by the addition of the dispersants in aqueous concentrations of PAHs and if it will affect the newly hatched mummichog fish survival and body length, etc. The addition of Corexit® 9500 increased the concentration of PAHs and the high-molecular weight hydrocarbons. Fish larvae exposed to DEWAF concentration of (0.0005 g/L; total PAH 479 ng/ml) exhibited the highest mortalities (98%). Increased concentrations of total PAH (r^2= 0.65, p= 0.02) and not with high-molecular weight hydrocarbons.

2.2 Toxicity of Dispersed Oil

Worldwide laboratory investigations such as research studies conducted by the Australian Maritime Safety Authority (2006) have provided a sound understanding of the toxic behavior of oil dispersants. Australian laboratory tests approved oil spill dispersants rate principally as "slightly toxic" to "nearly non-toxic" by the International Maritime Organization/ GESMAP classification system (IMO, 2002).

It must be recognized that, essentially the toxicity of dispersed oil is derived from the toxicity of the crude oil itself. In addition, applying acute toxicity testing of dispersed oil on a range of marine test species has indicated that the toxicity of dispersed oil generally does not initiate from the dispersant individually, but it originate in the more toxic fractions of the oil itself. Numerous studies like (Bobra et al., 1989; Tarzwell, 1971; Verriipoulos and Moraitiou-Apostolopoulou, 1982) have proven that

dispersed oil shows higher toxicity to aquatic organisms than oil or dispersant do if present alone in the marine environment. On the other hand, other studies have indicated that dispersant alone exhibited more toxicity than either dispersed or undispersed oil (Unsal, 1991). In the case of Exxon Valdez Oil Spill (EVOS), numerous and comprehensive field assessments and laboratorial studies have been conducted by academic and governmental researchers over the 30 years following the accident. Many of the insights originated from the EVOS regarding the long-term impacts of hydrocarbons on marine ecosystems have been challenged by research studies documented following EVOS. It was concluded that oil individually is considered to be persistent in the shoreline environments which tend to cause long-term injury to coastal organisms (Ott and Stanley, 1991). PAHs which are released from oil layers at increasingly slower rates with an increasing molecular weight will eventually lead to greater persistence of larger PAHs (Short et al., 2003).

2.3 Toxicity of Chemical Dispersants

The chemical composition of oil dispersants principally influences its toxicity to selected marine test species under controlled laboratory conditions, for instance: molecular structure of surfactants, chemical concentration, aromatic content, and the type of solvent involved. In terms of the factors affecting the toxicity of oil dispersants on aquatic species, the following examples provides suitable points to be considered such as: species type, species age, species developmental stage (growth), and duration of species exposure to the test chemical.

In the case of *Torrey Canyon* spill, extensive research has been conducted since 1967 when solvent emulsifiers (dispersants) were utilized to clean oil from the affected zone and it was proposed that the use of solvent emulsifiers was an efficient tool in combating oil spill by dispersing it with a no risk level to the aquatic habitats. It was concluded that earlier types of dispersants utilized in the *Torrey Canyon* spill exhibited broad differences in terms of its toxicity with dispersants which have been developed later. Dispersants applied in the *Torrey Canyon* spill demonstrated a LC_{50} (lethal concentration which affect 50% of species population) of 6 mg/l in respect of the brown shrimp (*Crangon crangon*) when using BP® 1002 dispersant which principally contains about 60-70% aromatics. The other type of solvent BP® 1100 exhibited an LC_{50} greater than 3.3 g/L and was based on non-aromatic constituents. Presently, the new dispersant

products are considered to be safer for use in the marine environment than the ones applied in the *Torrey Canyon* spillage (ROPME, 1998).

2.4 Water - Soluble Fractions

When crude oil is spilled on sea water and immediately diluted, a mixture termed "Water-Accommodated Fraction" (WAF) was developed and when chemical dispersant is applied on the oil slick afterward a mixture termed "Dispersed Oil Water-Accommodated Fraction" (DWAF) was developed. It has been concluded from several scientific evidences that DWAF is more toxic than WAF or chemical dispersant alone (Anderson et al., 1974; Wells et al., 1975; Holdway et al., 2000). As mentioned before, PAHs contain low-molecular weight hydrocarbons which are soluble in water column, so they are not persistent in sea water and they quickly diminish, but they tend to develop short term toxicity if present in high concentrations and will ultimately increase the toxicity of DWAF (Couillard et al., 2005).

2.5 Laboratory Toxicity Testing (Exposure Regime)

Although laboratory toxicity testing frequently represents less-than-ideal models of accurate environmental consequence, they are used as a vehicle to compare the various effects of several types of dispersants through testing diverse existing agents using standardized models (Singer et al., 1996). Some scientists ascertain that the application of dispersants in the open sea will decrease the acute toxicity effects of a dispersant on aquatic organisms due to the fact that dispersed oil tend to mix with a large body of water that is the sea. As a results, dispersed oil concentrations and exposures periods will be minimum in comparison to the situation if the dispersant is applied in a controlled laboratorial conditions. Certainly, there is a lack of standardized methods in the area of oil and dispersant toxicity testing and comparison of generated data is rather difficult (Markarian et al., 1995; NRC, 1989). As Singer et al., (2000) have indicated, the collected data are unfortunately not comparable because of the diverse analytical and toxicological methods followed. Since each country has its own unique marine ecosystems, the results of oil spills or use of chemical dispersants will surely vary among these ecosystems due to the varying ecological parameters such: sea water temperature, salinity, dissolved oxygen and additional weathering processes

within each ecosystem. Given the variability of crude oil, chemical dispersants and marine organism's types involved, the effects of these compounds are likely to be highly variable between geographical regions.

The debatable notion among aquatic toxicologists is the mere fact of whether or not oil dispersion enhances or reduces the exposure of marine organisms to the individual toxic components of oil (Mielbrecht et al., 2005; Ramchandran et al., 2004).

Latest information on combined toxicity of a mixture of compounds has demonstrated that the types of interaction exhibited by components of mixtures are chiefly dependent on the proportion of their concentrations in the mixture (Otitoloju, 2002; 2003). Hence, from previous experiences with the types of dispersants applied, it was demonstrated that dispersants have increased the toxic effect of oil when introduced to marine organisms and may possibly have caused a decrease in the toxic effect of oil or the mixture at different dispersal proportions (Otitoloju, 2005).

2.6 Crude oil and Developmental Abnormalities in Fish

Fish early-life stages are considered to be the most sensitive life stages to oil contamination and other sources of pollution in the marine environment, and hatched larvae which drift in the sea currents can reach oil spills and thus can be affected. Various types of xenobiotics like: petroleum hydrocarbons, heavy metals, pesticides, and fluctuations in water quality parameters such as salinity, dissolved oxygen, temperature can be the major causes of fish larval developmental abnormalities like: yolk-sac edema, skeletal deformity, craniofacial deformity, retarded growth, pericardial edema, impaired gonadal development, body shortening, and hemorrhage (Witeska and Lugowska, 2004; Incardona et al., 2004;). The effects of seven nonalkylated PAH compound containing 2-4 rings such as: naphthalene, fluorine, dibenzothiophene, phenanthrene, anthracene, pyrene and chrysene on zebrafish development were examined and it resulted in gross normal anatomic features when treated with naphthalene, anthracene or chrysene. But, developmental abnormalities such as dorsal curvature of the trunk and tail and significant growth reduction partially of the head were prominent when larvae were treated with with fluorine, dibenzothiophere, or phenanthrene. More treatment with other PAHs resulted in other developmental deformities like mild-to-severe pericardial and yolk sac edema, and a less-pronounced dorsal curvature Incardona et al. (2004).

Heavy metals which become bioavailable in the water column have the ability to accumulate in fish gonads, affect gamete production and have an adverse effect on embryo-larval development ultimately causing developmental anomalies which can result in a reduced number of larvae (Jezierska et al., 2009).

2.7 Hypothesis

The hypothesis of the proposed research study aims to test:

1) If Kuwait crude oil WAF (KCO WAF) by itself is toxic to marine fish embryonated eggs and larvae.

2) If the addition of oil dispersants Corexit® 9500, Corexit® 9527 and Slickgone® NS dispersants (CE-WAFs) individually to Kuwait crude oil will result in enhanced/ similar or lower toxicity compared to that of KCO WAF against selected marine fish species embroys and/or larval stages.

3) If the embryonated eggs/larval stages are more/less or equally sensitive to the toxicity of KCO WAF or CE-WAFs.

4) If the selected fish species: sea bream, yellow-fin sea bream, orange-spotted grouper and mullet have similar/ dissimilar sensitivities to the toxicity of KCO WAF or CE-WAFs.

5) If the addition of oil dispersants Corexit® 9500, Corexit® 9527 and Slickgone® NS dispersants (CE-WAFs) individually to Kuwait crude oil compared to that of KCO WAF will result in an increase in the concentration of the following: BTEX, PAH's, aliphatics, TPH, absorbance and fluorescence.

6) If exposure to KCO WAF can induce any sublethal effects such as fish developmental abnormalities in fish larval stages.

2.8 Aims of the Study

1) The three chemical mixtures are aimed to be characterized for the following devised chemical compounds:

A. Benzene, toluene, ethylbenzene, and xylene (BTEX)

B. Polycyclic aromatic hydrocarbons (PAH)

C. Aliphatic Hydrocarbons

D. Total petroleum hydrocarbons (TPH)

E. Absorbance

F. Fluorescence

2) Toxicity tests (bioassays) will be conducted through exposure of selected life stages of fish species cultured in Kuwait to the prepared chemical mixtures following standard universal guidelines. The following are the test species which will be considered for the proposed research study:

A. Sobaity-sea bream (*Sparidentex hasta*)

B. Hamoor-orange-spotted grouper (*Ephinephelus coicoides*)

C. Meid-Mullet (*Liza Klunzingeri*)

D. Shea'am-Yellow-Fin sea bream (*Acanthopagrus latus*)

3) The mortality of marine fish (egg and larvae) tested will be recorded and calculations of LC_{50} (lethal concentration which affects 50% of the fish population) will be conducted in addition to other relevant statistical tests to gain a better understanding and interpretations of the collected data.

4) Sublethal effects such as developmental abnormalities in fish larvae after exposure to Kuwait crude oil water-accommodated fraction will be recorded.

5) Recommendations will be provided to regional and local authorities in Kuwait. The most permissible concentrations of crude oil, oil dispersants and dispersed oil needed to be applied in case there is an oil spill incident in Kuwait Territorial Waters will be indicated, with respect that those permissible concentrations will not cause an adverse effect on marine organisms.

Chapter Three

CHEMICAL CHARCTERIZATION OF WAF AND CE-WAF OF KUWAIT CRUDE OIL AND THREE OIL DISPERSANTS

3. 1 Introduction

Kuwait is considered a prominent country among other oil producing countries in the world. Kuwait crude oil in nature is unique from chemical composition point of view and the oil type exported from Kuwait is a blend of many different oil types. Blended Kuwait crude oil which is of an export quality fundamentally depends on numerous factors such as the availability of different crude oils, consumer specifications, the refining process requirements and other relatively economic factors. During the process of exporting crude oil to other regions of the world, accidental oil spills are common phenomena. In the case of an oil spill, oil particles normally tend to dissolve in the water column because of wave and tidal actions, therefore the effect and composition of this soluble compounds in crude oils has been the subject of many studies (Boylan and Tripp, 1971; Anderson et al., 1974; Lee et al., 1974; Mackay and Shui, 1976; Shui et al., 1990). The studies have demonstrated that the water-soluble fraction of crude oil is essentially a complex mixture, which consists of nitrogen- and sulfur containing heterocyclic compounds, pentane, polycyclic aromatic hydrocarbons, phenols, and benzene, toluene, ethylbenzene, and xylene (BTEX) compounds. And, since the only fraction of a chemical product that enters the water column water-accommodated fraction (WAF) has the potential to produce noticeable toxic effects on aquatic organisms individually or with the addition of oil dispersants, this test media was characterized in terms of its chemical constituents.

WAF preparation methodology; is a well-documented in literature since it serves many purposes in marine environmental research of which we are interested in this research is the aquatic toxicological evaluation of WAF/CE-WAF. Preparation of a test media for toxicological investigation studies should be performed with a complete consideration of the particular objective of the desired toxicological analysis in mind, since the preparation method can influence the composition of the aqueous phase associated with the chemical substance being prepared (Lockhart et al., 1987; Shui et

al., 1988; Bennet et al., 1990; OCED, 2000). One of the most essential requirements of a test media preparation is that it should be environmentally realistic and reproducible over time and between laboratories. In addition, principal characterization and understanding of test media behavior is essential to ensure the suitability and reproducibility of the chemical solution specifically in toxicity studies. Moreover, since the only fraction of a chemical product that enters the water column has the potential to produce a noticeable toxic effects on aquatic organisms, therefore; test media exposures should be quantified accordingly in determining toxic concentrations, specifically when comparing various media preparations or different chemical compounds (Singer et al., 2000). In this research study, Kuwait crude oil water-accommodated fraction (WAF) and Kuwait crude chemically enhanced water-accommodated fraction (CE-WAF) that is oil plus three individual oil dispersant formulations (Corexit® 9500 & 9527, and Slickgone® NS) were chemically characterized for total petroleum hydrocarbons (TPH), polycyclic hydrocarbons (PAHs), BTEX, and aliphatic compounds to further understand the behavior of all of these chemical fractions of WAF solution after its preparation.

Fundamentally, there are three established methods of preparing aqueous test solutions associated with crude oil: A) water-accommodated fraction – (WAF), and B) water-soluble fraction – (WSF), and C) chemically enhanced water-accommodated fraction (CE-WAF). There is a fairly extensive literature published regarding the technical advantages and disadvantages of selecting either one of the three methods and their definitions are clearly described in Singer et al., (2000).

3. 2 Water-Accommodated Faction (WAF)

WAF is a laboratory-prepared test medium derived from the low mixing energy of a poorly soluble test material such as petroleum and/ or another oil product without the formation of a vortex. This medium should be in essence free of any particles of bulk material (Aurand and Coelho, 1996; Coelho and Aurand, 1997). Others like Calow (1998) described WAF as a medium which only contains that fraction of a product that is retained within the aqueous phase once any mixing energy has been removed and the fraction of the product may be present either in solution or as a stable mixed emulsion.

3. 3 Water-Soluble Fraction (WSF)

WSF can be defined as a chemical solution obtained after filtration of the aqueous medium which was mixed for a specific period of time such as 20 to 24 h thus, guaranteeing that most of the particulate oil was removed from the medium. The mixture contains dissolved hydrocarbons, oil droplets and metallic ions and those constituents which are partitioned into the solution make the WSF (Kauss and Hutchison, 1975; Ali and Mai, 2007).

3. 4 Chemically Enhanced Water-Accommodated Fraction (CE-WAF)

CE-WAF is a technical term associated with the oil/dispersant mixtures. It can be defined as a laboratory prepared-medium obtained from a standard 20-25% vortex mixing of a test material such as crude oil and a chemical dispersant by which a relatively constant population of bulk material droplets (100 μm in diameter) is present (Aurand and Coelho, 1996; Coelho and Aurand, 1997). The term CE-WAF was selected because the true role of oil dispersant is to enhance the accommodation of bulk oil in the water, which is in its turn increases its functional solubility (Singer et al., 1998). It's essential while intending to prepare such test medium to note that mixing energy should be adequate to create a vortex for dispersant/oil interactions consistent with the purpose of using the oil dispersant for instance, toxicity testing (Singer et al., 2000). What should be noted here is that CE-WAF is prepared in same manner, as WAF solution, but in the latter, oil dispersant is added to it as will be discussed in the preparation methodology section below. Couillard et al., (2005) have used another term which describes this test medium as dispersed oil water-accommodated fraction (DWAF).

Principles of characterizing and understanding the behavior of chemically prepared WAF should be clarified and described in terms of partitioning effects. In a sense that if differential partitioning effects of a product constituents between a product such as crude oil and the aqueous phases are ignored, then increasing the product-to-medium ratio will increase the ability for product constituents to enter into solution in the aqueous phase up to the limit of their aqueous solubility. Moreover, by increasing

the time and energy of mixing during the preparation of test media, the potential for a crude oil product to emulsify will definitely increase.

Through the years, the term water-accommodated fraction (WAF) as opposed to the water-soluble fraction (WSF) term became favorable among researchers and investigators of oil spills, and was considered to be a more technically correct and appropriate term. A proper justification for the selection of this term is that chemically prepared solutions do not necessarily go through complete steps to guarantee that all possible particulate whole oil has been removed through ordinary removal methods such as centrifugation and filtration, in other words; oil droplets are not removed from solution like in the case of WSF (Girling, 1989; Bennet et al., 1990; Girling et al., 1992; Singer et al., 2000). In addition, whichever methods are applied will cause a loss in the chemical nature of the test media or induce prominent changes in its consistency.

After reviewing several preparation methodologies during the course of this research, it is recommended that the most appropriate test media preparation procedure should be conducted in such a manner that generates an environmentally realistic test media with the need for a media type that has a highly reproducible chemical composition (Singer et al., 2000). In literature, there exist numerous methodologies describing test media preparation techniques, in which, test vessel size, mixing energy, mixing duration are thoroughly discussed and illustrated (Girling, 1989; Ali et al., 1995; Saeed and Al-Mutairi, 2000; Singer et al., 2000; Couillard et al., 2005). Previously reported data on test media procedures have some shortcomings, many, for example, compare the effects of crude oil individually or with addition of oil dispersants but lack of analytical verification. It is extremely important to evaluate the prepared test media specifically in the case of oil alone and/or oil plus chemical dispersant because the principal purpose of the use of oil dispersant is to increase the entry of oil into the surrounding seawater, thus modifying the exposure nature. Moreover, since the only fraction of a chemical product that enters the water column has the potential to produce a noticeable toxic effects on aquatic organisms, test media exposures should be quantified accordingly in determining toxic concentrations, specifically when comparing various media preparations or different chemical compounds (Singer and Al-Mutairi, 2000).

3. 5 Review of Water-Soluble Fraction (WSF) Literature in Kuwait

Since WSF term was widely used in the 1970s and 1980s, most of the research conducted in relation to Kuwait crude oil emphasized its analysis on WSF (water-soluble fraction) rather than WAF (water-accommodated fraction), as WAF became more popular in the 1990s and in the 2000's. Many researchers have reported the composition of polycyclic aromatic hydrocarbons (PAHS) in the WSF of Kuwait crude oil. Boylan and Tripp (1971), for example, have described the presence of compounds like naphthalene and its methylated derivatives. Murray et al., (1984) reported noticeable levels of naphthalene at low levels using a micro-extraction detection technique. Moreover, Anderson et al., (1974) discussed the chemical aspects of WSF including some significant levels of PAHs. Ali et al., (1995) has described in detail PAHs composition in the WSF of Kuwait crude oil and later, Saeed et al., (1998) have in detail investigated the composition of volatile organic compounds (VOC) and PAHs in the WSF of Kuwait crude oil at various temperatures and in Arabian Gulf high salinity seawater. Furthermore, Saeed and Al-Mutairi (2000) have described and compared the composition of polycyclic aromatic hydrocarbons present in the WSF's of ten different crude oils in addition to Kuwait crude oil export quality.

3. 6 General Review of WSF in Literature

In the case of an oil spill, oil particles normally tend to dissolve in the water column because of wave and tidal actions, therefore the effect and composition of these soluble compounds in crude oils has been the subject of many studies (Boylan and Tripp 1971; Anderson et al., 1974; Lee et al., 1974; Mackay and Shui, 1976; Shui et al., 1990). The studies have demonstrated that the water-soluble fraction of crude oil is essentially a complex mixture, which includes the following chemical compounds: nitrogen- and sulfur containing heterocyclic compounds, pentane, polycyclic aromatic hydrocarbons and phenols. Primarily, aromatics constitute the major class of hydrocarbons, which exist in WSF, and mononuclear aromatics represent approximately 98% of the total WSF (Carls and Rice, 1990). In addition, BTEX (benzene, toluene, ethylbenzene and xylene) constitutes approximately 88% of the total WSF.

3. 7 Review of Test Media Preparation Methodology

It is imperative to demonstrate the variations in methodologies regarding the preparation of WSF, WAF, and CE-WAF. Preparation methods of the test media intended to be used in toxicity testing against aquatic species have been debated extensively in the literature. Each method has a specifically defined purpose that is, producing a test media which replicates natural conditions and has a relative toxicity in the case of accidental oil spill. The development of the preparation method needs to encompass a test solution, which consists of crude oil and oil dispersant mixed together as observed in natural environment with same effects. Recently, the Ocean Studies Board (OSB, 2005) debated selected methods for the preparation of WAF, which are selected for use in this research. The Chemical Response to Oil Spill Environmental Research Forum (CROSERF) protocols have recommended preparation of toxicity test solutions by variable loading using a series of decreasing concentrations of applied oil and oil dispersant (Barron, 2003). Baron and Ka'aihue (2003) have proposed the use of a single oil loading concentration, which consists of water loading rate and the preparation of test solutions using a range of dilutions of the stock solution prepared as proposed by United Nations Environment Program (UNEP, 1989) which have been used by recent experimental studies. As previously discussed, the selection of the best method for WSF, WAF or CE-WAF eventually depend on the specific intention of the study and what questions needs to be answered. For instance, WAF/WSF/CE-WAF is prepared in some cases to simulate natural situations occurring during the oil spill (Hokstad et al., 2000). Also, to determine which fraction of the oil (dispersed oil particles or/ dissolved oil and dispersants) can be responsible for causing a toxic effect on marine biota (Bobra et al., 1989). In addition, test chemicals can be prepared to evaluate the weathering behavior of crude oil and to predict the changes in its concentration and composition in a short term period following the oil spill incident (Wang and Fingas, 1994). Many researchers like Singer et al., (2001a) have argued a method utilizing variable oil loading and they have justified their choice on the basis that this preparation method is more relevant to natural field conditions, since spilled oil slicks have particular characteristics, for instance, they tend to be dynamic and its size, thickness and their shape is continually changing.

3. 8 Comparison Between Kuwait Crude Oil WAF and CE-WAF

Since the primary objective of this research is to determine at what oil-to-water loading ratio of WAFs and CE-WAFs are considered to be toxic to marine organisms, preparation methods should reflect a relative answer to this question. Similarly, Barron and Ka'aihue (2003) support a variable dilution preparation method of WAF and CE-WAF for testing oil dispersant that standardizes the oil: water ratio, which provides a consistent chemical concentration in a test-series for each oil-dispersant combination. This approach in selecting a preparation method of WAF (CE-WAF) for toxicity testing will be more appropriate in ultimately assisting the investigator in obtaining a more definitive answer for the question raised by many researchers which is "At what dilution is a given oil: water ratio of WAF(CE-WAF) toxic?".

Overall, it has not been finally demonstrated that either methods would simulate the temporal dilution of dispersed oil under actual oil spill conditions. In this research WAF was prepared by variable oil loading using a series of decreasing concentration of applied oil and dispersant in seawater as described by CROSERF protocols (OSB, 2005), also. WAF was prepared by serial dilutions of single oil loading according to UNEP (1989).

3. 9 Single Concentration Oil loading Vs. Variable Oil Loading

UNEP (1989) recommend the use of single oil loading when preparing water-accommodated fraction (WAF) of crude oil, which was done in unreported data (chemical standardization experiments). And the Chemical Response to Oil Spill Research Forum (CROSERF) protocols (NRC, 2005) recommend variable oil loadings using a series of decreasing concentrations of applied oil and dispersant in the sea water. Singer et al., (2000) methodology was adopted to prepare test media for WAF and (CE-WAF) and test conditions. In the chemical standardization experiments performed in our laboratory, WAF prepared from single Kuwait oil loading and subsequently making serial dilutions of the WAF and CE-WAF solutions for toxicity exposure; provided results which were consistent and comparable. Hence, single oil loading of 1 g KCO/L filtered seawater (2 g KCO/2L filtered seawater) was selected for the preparation of

WAF for subsequent experiments. As for the CE-WAF preparation, 10% of the oil dispersant 0.1 g (0.2 g) was selected and added over the oil layer (Table 3-1).

Table 3-1: Experimental settings for WAF preparation

Experimental Conditions for WAF Preparation	
Seawater Type	Natural seawater filtered through 0.45µm filter paper
Water Quality	Dissolved Oxygen, Temperature, Salinity, pH, Conductivity
Vessel Size	2 L glass aspirator bottle
Volume of Seawater	2 L constant depth 16.5 cm
Head Space	Determined 340 ml (constant)
Crude Oil Type	Standardized quality of Kuwait Crude Oil Export Quality API 31.8
KCO Loading	Concentrations indicated in text
Stirrer Bar Size	2.5 cm (constant)/ 3.5 cm
Mixing Speed	300 rpm-Bunsen® stirrer vortex 2.5 cm (20-25% vortex)
Mixing Duration	16-24 h (constant)
Settling Duration	3 h (constant)
For Dispersants	10% determined
KCO/Dispersant Ratio	10:1 (oil:dispersant)

Adopted from Singer et al., (2000).

Others such as Cotou et al., (2001) have tested the individual toxicity of oil dispersants without the presence of crude oil. A stock solution was prepared by diluting concentration of 1 mL of surfactant-based oil dispersant (Finasol® OSR-5) into natural seawater (1L) that was filtered only once using Whatman® filter size 0.45µm before use. The chemical mixture was blended for 30 minutes at an approximate mixing speed of

2000 cycles per minute and eleven serial dilution concentrations were prepared of the oil dispersant concentrations (10 to 600 mg/L) and were tested.

In addition, Stakėnienė et al., (2004) have tested the toxic effect of another type of chemical dispersant Simple Green® (SG) which was used in Lithuania after the oil spills incident into the Baltic Sea in 2001. The swirling flask method (Fingas et al., 1987) was adopted using a ratio of 4 portions of crude oil and 1 portion of Simple Green® oil dispersant, which was chosen following the recommendations of the United States Environmental Protection Agency (USEPA) on oil spill liquidation in open waters. Simple Green® solution was poured onto the film of oil which was poured on the water surface and the mixture was stirred for 15 minutes then the solution was left to stand for another 15 minutes (www.consumer.simplegreen.com, 2005). Moreover, WAFs have been prepared according to Singer et al., (2000) in which they stirred crude oil with dechlorinated municipal water at a ratio of 1:9 (oil-to-water) in a sealed container for 18 h at a temperature of 18°C and this ratio of was selected on the basis that it will produce a maximum TPH content in the water column (Gagnon and Holdway, 2000). The vortex was adjusted to no more than a third of the height of the prepared chemical mixture from the oil-water interface and the mixture was allowed to settle for 1 h to ensure a phase separation of water and oil.

As for the chemically enhanced water-accommodated fraction (CE-WAF) it was prepared by mixing crude oil and water in the same ratio and test conditions which was used for the preparation of WAF solution. Then, Corexit® 9500 oil dispersant was delivered to the surface of the oil-water mixture at a ratio of 1:20 oil: dispersant according to Gilbert (1996) and allowed to stir for an additional one hour and then to settle for one hour.

3. 10 Review of WSF Preparation Methodology

Aquatic toxicological research has chiefly dealt with the determination of crude oil toxicity, focused particularly on water-soluble fraction because it's the fraction of crude oil that enters marine environment easily and can induce immediate and an acute damage on marine organisms. However, the remaining components of this mixture eventually are incorporated into the water column (Martínea-Jerónimo et al., 2005). A variety of methods have been adopted by researchers over the years for the preparations

of a water-soluble fraction (WSF) of oil in water such as the methods developed by (Lockhart et al., 1984; Shui et al., 1990; Paine et al., 1992). Others like Phatarpekar and Ansari (2000) have prepared a water-soluble fraction by adding one part oil to nine parts water, that is 50 g of crude oil was added to 450 ml of reconstituted hard water. Tsvetnenko and Evans (2002) studied factors which affect petroleum hydrocarbon solubilization in aqueous systems during preparation of test solutions, intended for toxicity studies. In addition, the collective individual influences of mixing speed, mixing time, volume/interface ratio, and hydrocarbon concentration in WSF were examined thoroughly. Test conditions of different preparations methods for WSF and WAF preparations are illustrated and compared in (Table 3-2).

Table 3-2: Summary of selected previous studies on the preparation methodology and quantification of WSF and WAF

No.	Author & Year	Type of Crude used	Oil : Water Ratio	Preparation Methodology	Stirring Time (h)	Water Temperature (°C)	Salinity ‰	Settling Time (h)	Method of Quantifying Total Compounds in WSF	Concentration of Total WSF µg.ml $^{-1}$
1	Anderson et al., 1974	South Louisiana, Kuwait	1:9	Magnetic stirrer bar was used to slowly stir the seawater in a 5 gallon Pyrex bottle, which was capped with aluminum foil to minimize evaporation. The vortex was 25% of the distance to the bottom of the bottle. After settling time the water phase was withdrawn and utilized instantly.	20	20±2	15-20	1-6	IR, measured in CCl_4 extracts (API method no. 733-58)	South Louisiana crude = 19.8 Kuwait crude = 10.4
2	Burwood & Speers, 1974	Middle East. s.g(20°C) =2.00 Wax(%wt) = 5.00	1:50	Artificial sweater was stirred slowly by a magnetic stirrer bar in a conical flask, which was covered with cotton wool plugs. A tap filter was used to siphon the water phase at the bottom of the flask.	6 hour to 4 weeks	20±2	38	NR	UV, measured in octane extracts in the region of 240-280 nm, calibrated with standard solution prepared from benzene in iso-octane.	11-23 expressed arbitrarily in terms of benzene equivalent.

3	Winters et al., 1977	Kuwait, Southern Louisiana Venezuela, Alaska, and Fuel oil	1:8	Sewater was stirred in a sealed bottle at a rate in which a formation of an emulsion was avoided and the water phase was siphoned by using a stopcock situated at the bottom of the sealed bottle.	24	25	NR	Several minutes	Continuous liquid-liquid extraction with benzene, dried by evaporation at room temperature then weighed, redissolved and analyzed by Gas Chromatography.	Not indicated for the selected crude oils.
4	Cladwell et al., 1977	Cook Inlet crude oil	1:100	Magnetic stirrer bar was used to slowly stir the seawater in a 5 gallon Pyrex bottle, which was capped with aluminum foil to minimize evaporation. The vortex was 25% of the distance to the bottom of the bottle. After settling time the water phase was withdrawn and utilized instantly. (Anderson et al., 1974 Method).	20	13-	29-34	4	UV measured at 221 no wavelength in hexane extracts.	0.0083± 0.0019 expressed as naphthalene equivalent.
5	Blackman and law, 1980	Kuwait	1:9	Seawater was stirred in a glass aspirator bottle with selected size between (5-15 L) which was tested. The bottle neck was closed but not sealed, vortex was 25% if the water column depth. Water phase was siphoned from the bottle tap and filtered using pressure through 0.2-0.45 μm filter	24-72	19.5± 1.5	33.5	0-1	Two Methods: 1) IR, absorbance was measured at 2930 cm/l corresponding to the stretching frequency of C-H bands in aliphatic CH_2^- groups. 2) UVF, measured at two wavelengths 310	0.5 – 1.00 oil equivalent. 1.1 – 5.8 oil

				paper.				nm excitation and 360 nm emission in solvent extracts. The quantification for the above two methods was performed with respect to the fresh crude oil used in WSF preparation.	equivalent.
6	Busdosh, 1981	Prudhoe Bay	1:1000,000 1:100,000 1:10,000 1:1000 1:100 1:10	Seawater was stirred by using Teflon-coated magnetic stirrer bar. The mixture was allowed to stand for phase separation and the WSF was withdrawn from 5 cm below the oil slick.	-	5	27	12		
7	Pearson et al., 1981	Prudhoe Bay	1:9		20	20	24	4	By GC for other hydrocarbons present after acidifying the WSF and extraction with hexane. In addition helium equilibration GC was used for the monoaromatics.	24.05
8	Østgaard and Jensen, 1983a	Ekofisk	1:20	Seawater was sterilized by autoclaving, which was stirred at 120-170 voltage by magnetic stirrer in a 5 L glass bottle closed with a silicone stopper. Water phase was siphoned by pumping through glass/silicon rubber tubing situated below the level of oil layer and collected in a sterilized brown flask. The bottle was	10 days	14	25	NR	By three methods: 1) Extracted with DCM for the volatile fraction, then dried with Na_2SO_4 and concentrated to 300 µl, then analyzed by gas chromatography. 2) Direct fluorescence analysis at excitation wavelength 230 &	C_{7+} fraction was 4.6 UVF results were expressed in fluorescence intensity units: 1.200±4 at λ excitation/emission = 230/335 nm 806 ± 5 at λ excitation/emission = 265/300 nm.

				completely filled.				265 nm and emission wavelength 300 & 335 nm for: naphthalene and phenol components respectively. 3) Head space analysis for the highly volatile fraction by using benzene-d_6 as internal standard.	
9	Lockhart et al., 1984	Norman Wells, Kuwait	1:20 to1:1000	Seawater was stirred vigorously on a magnetic stirrer plate, the mixture was allowed to settle until phase separation was achieved then the SWSFs were withdrawn from the bottom of the mixing vessel.	2	NR	NR	48	By gas chromatography using headspace analysis for the most of the volatile fraction, in addition, solvent extraction analysis was used for the less volatile materials.	Norman Wells = 60.1 Kuwait = 35.3
10	Michel and Case, 1984	Platform Holly, Monterey Formation	1:25	Nitrogen gas was used to flush the headspace above the oil layer in a 2 L glass aspirator bottle. Seawater was stirred with 40 mm stir bar to achieve a 7 mm deep vortex. SWSF was flushed after allowing the mixture to stand after mixing duration through the stopcock into clean containers with no headspace.	48	NR	NR	1	Gas chromatography was used to quantify volatile compounds present in the SWSF using a gas/tenax trap method optimized for the recovery of benzene.	16.3 for benzene, toluene, ethylbenzene and xylene (BTEX).
11	Maher, 1986	Barrow, Copper, Arabian Light, Qatar	1-3 g oil: 400 ml seawater, specific ration was not specified	Seawater which was sterilized by steaming was stirred on a magnetic stirrer plate to obtain 0.5 cm vortex. A tube situated below the oil layer was used to	24-72	20	NR	10-15 minutes	UVF was used at 300 nm excitation wavelength and maximum emission wavelengths were determined by scanning between 310-	Results were as minimum (0.45 µm filtrates) – maximum (filtered): Barrow = 0.28-3.1

			for each oil.	drain the SWSF sample which was later measured unfiltered and filtered using 0.45-1.0 µm pore size filter paper.					390 nm. Quantification was performed with reference to the respective oils.	Copper = 0.39-2.6 Arabian Light = 0.14-1.03 Qatar = 0.22-1.9
12	Glamuzina et al., 1990	Iraq	1:9	Anderson's method et. al., 1974	20	18	38	1	UV was measured in hexane extracts at 221 nm, standardized with naphthalene.	0.189 expressed as naphthalene equivalent.
13	Paine et al., 1992	Hibernia	1:24	Seawater was filtered and sterilized with ultraviolet light and stirred at constant speed with paint stirred powered by a mounted drill motor. The 4 L glass jar vessel containing the mixture was inverted and left to separate. Oil which didn't dissolve in the water phase rose to the top and the WSF was siphoned from the bottom of the glass flask.	10	NR	NR	18	UV, measured at selected wavelengths (not indicated) and quantification was performed by using a calibration curve.	5.43 (range 3.96-6.53, SD 0.67)
14	Sophia and Blasubramanian, 1992	Kuwait	1:9	Anderson's method et al., 1974	20 hr	20 ± 2	NR	1-6	UVF, no details of quantification method was indicated.	12.72
15	Ali et al., 1995	Unrefined Kuwait crude oil	1: 100	Seawater (900 ml) was filtered with filter paper pore size 0.45 µm and poured into 1 L glass reagent flask, Teflon-coated magnetic stirrer bar was added and the flask headspace was flushed with nitrogen gas to eliminate any air	10 days	25	33 ppt	No settling duration	Comparison between three methods: 1)UVF 2) DOC 3) GC/MS	3-4 µg ml^{-1} 3-4 µg ml^{-1} 9 µg ml^{-1}

				present in the vessel. A syringe through Suba added crude oil (9 ml). Seal and a U shaped stainless steel tube were inserted in the Suba.Seal into the water below the oil layer to collect the WSF. The mixing vessel was placed in a water bath with a controlled temperature to bring the water temperature to 25°C. Mixing was performed at 150 rpm					
16	Singer et al., 2000	Prudhoe Bay crude oil	0.01, 0.1, 1 and 10 g /L (ratio of oil to seawater was expressed as a mass per volume)	Anderson's method et al., 1974. Testing of various parameters like: mixing vessels sizes, seawater volume, headspace volume, surface and area/ volume ratio.	6, 12, 18, 24, 36, 48, 60 and 72 h	NR	33 ppt	18	Total carbon analysis in addition to flame ionization gas chromatography (GC/FID)	
17	Saeed and Al-Mutairi, 2000	10 different crude oil types from Kuwait	150 ml oil : 1.5 L seawater	Ali et al., 1995 method were 2 L bottle which contained seawater and 2 cm stirring bar kept in a water bath, which was placed on a magnetic stirrer. Oil was introduced by a syringe to the top of the water layer. No vortex was initiated. Nitrogen gas was used to drain the WSF.	24	± 0.1°C	39 ppt	No settling duration	Gas chromatography/ Mass spectrometry operated in single ion monitoring (SIM) mode was used to analyze concentrated extracts in which WSF was extracted with hexane/ dichloromethane.	PAHs in WSF ranged from 171-2176 µg/l. oil from northern oil fields contained higher PAHs levels. Naphthalene and its homologs formed the bulk of PAHs.
18	Tsvetnenko and Evans, 2002	Different crude oils produced in the North	9 distilled water : 1 oil	Anderson et al., 1974, experiments were conducted to examine the influence of	24-600 h	25	35 ppt	NR	By purge and trap GC/MS for the carbon range of C6-C9and solvent extraction with	CO_{21} = 5 mg/l CO_{34} = 16 mg/l

		West shelf of Western Australia known as CO_{21}, CO_{34}, and CO_{48}		agitation rate, flask configuration (volume/interface surface ratio) agitation time on TPH concentrations in WSF preparations.				GC/FID for the carbon range C10-C36. UV-spectroscopy was used to measure TPH concentration in water samples.	CO_{48} = 9.2 mg/l
19	Barron et al., 2003	Weathered Alaskan North Slope crude oil (ANS)	0.3 ml oil : 32 L seawater	WAF was prepared in 50 L fiberglass tanks which were partially filled with 32 L of seawater filtered through 1-µm polyester filters. A motor-driven paddle shaft extended until 2.5 cm above the tank bottom with a speed of 600 rpm produced vigorous mixing which resulted in a long vortex from the water surface down to the paddle. Oil was added near the margin of the vortex by pipette. Mixture was drained from the bottom of the tanks through a presoaked 1-µm polyester filter and WAF sample was collected in 4 L glass jar.	12	NR	NR	1 h	Water samples were extracted with dichloromethane after adding six internal standards. WAF analysis was done by HPLC and GC/MS and total PAH concentrations were calculated by summing concentrations of the individual PAHs.	PAH of WAF prepared from 3.000 µl oil was 87 µg/l as naphthalene was the dominant compound. While addition of dispersant to WAF resulted in a PAH of 440 µg/l.

20	Fuller et al., 2004	Arabian medium crude oil	NR	Oil was added to 2 or 4 L glass aspirator flask with a gastight syringe and delivered on premeasured dilution water. Flask was sealed with Teflon stoppers and placed on a magnetic stirrer plate in which its speed was adjusted to obtain 25% vortex of the total water column depth. All flasks were covered with aluminum foil to prevent photoxidation of the test media during the test.	48	25± 2°C	20 ppt	No settling duration	Gas chromatography-mass spectrometer method to analyze nonvolatile hydrocarbons both saturated (*n*-alkanes) and aromatics (naphthalene, pyrene, chrysene etc.).	Total TPH = 0.00081 mg/l (predicted aqueous concentration), and 0.00132 mg/l (solubility mg/l)
21	Couillard et al., 2005	Weathered Mesa light crude oil	0.2-1.0 g oil : 1.8 L seawater	Method by CROSERF adopted by Singer *et. al.*, 2000. Crude oil was pre-weathered by sparging with air at room temperature for 24-48 hr until 20% of the oil volume had been lost. Seawater was filtered through 0.2 µm filter paper. Low mixing speed selected which didn't produce a vortex. WAF of crude oil was prepared in addition to WAF after the addition of oil dispersant (DWAF).	24	NR	30 ppt	No settling duration	HPLC and GC were used to analyze concentrated extracts of PAHs and high-molecular-weight PAH with three or more benzene rings.	PAHs in WAF of oil loading 0.2-1 g/l ranged from 190-243 ng/ml. while PAHs in DWAF prepared from oil loading of 0.05-0.5 g/l ranged from 136-479 ng/ml.

22	Lavarías et al., 2006	Punta Loyola light crude oil	1 oil : 100 freshwater (v/v)	Crude oil was stirred in a 10 L Stainless steel mixing vessel with a mechanical stirrer at low speed and WSF was collected daily from the bottom drain	24	4°C	NR	48	GC/MS method in which specimen compounds were identified by comparison of retention times with those of external standards and with WSF.	WSF was mainly composed of single ring aromatic hydrocarbons (composition details described in Lavarías et al., 2004)

3. 11 WSF and Toxicity Testing

Water-soluble fractions prepared to be used for toxicological assessment of crude oil have further limitations in a sense that many methodologies have been implemented with differences in crude oil-to-water volumes and proportions, in water quality conditions such as temperature, and in duration and type of mixing. These disparities can lead to enormous variations in the available data related to WSF and thus, render comparison between preparation methodologies rather difficult (Singer et al., 2000). Hence, standardization of preparation methods such as analytical, biological and chemical is a necessity when conducting similar studies (Martínea-Jerónimo et al., 2005). What should be clearly noted is that toxicity of petroleum products should be assessed by testing the prepared WSF, which should be containing the maximum possible fraction of dissolved hydrocarbons (Tsvetnenko and Evans, 2002).

3. 12 Materials and Methods

3. 12. 1 Kuwait Crude Oil (KCO)

Kuwait Export Crude Oil (API-3.18) was procured from Petroleum Research Center (PRC) of Kuwait Institute for Scientific Research (KISR) and stored in a 2L amber bottles with no head space in room temperature (26°C) in dark. The technical specifications of this crude oil are: gravity (30.18º), density (at 15º is 0.8744 g/ml), sulphur content (2.6 % weight), viscosity (at 20º 17.38 cSt), and Conradson Carbon Residue (CCR- 6.2 % weight).

3. 12. 2 Oil Dispersants

Chemical dispersants samples were procured from original manufacturers Onedo Nalco Ltd. (2005), United Kingdom (Local agent Bobyan Shipping & Marine Services) and Dasic United Kingdom (2007) (local agent Middle East Chemical Manufacturing Co.).

3. 12. 2. A Corexit® EC 9500A

The product contains Propylene glycol 1-5%, w/w; Distillates, petroleum, hydrogenated light 10-30%, w/w; Organic sulfonic acid salt (proprietary) 10-30%, w/w. This product is harmful by inhalation on repeated or prolonged exposure and may cause irritation of respiratory tract eyes and skin. If swallowed, it may cause nausea and vomiting and CNS depression. The organic portion of this preparation is expected to be biodegradable and component substances have a potential to bioconcentrate. Ecotoxicological studies rated this compound as slight toxic with Artemia 48 h LC_{50} equivalent to 0.021 g/L and (*Acartia tonsa*) 48 h LC_{50} 0.034 g/L (Nalco Material Safety Data, 2005). The material, safety and data sheet is included in Appendix A-1.

3. 12. 2. B Corexit® EC 9527A

This product contains 2-butoxyethanol 30-60%, w/w; Propyleneglycol 1-5%, w/w; Organic sulfonic acid salt (proprietary) 10-30%, w/w. This product is harmful by inhalation, in contact with skin and if swallowed, irritating to eyes and skin. The organic portion of this preparation is expected to be readily biodegradable and component substances have a low potential to bioconcentrate. However, ecotoxicological effects are not known since toxicity studies have not been conducted on this product (Nalco Material Safety Data, 2005). The material, safety and data sheet is included in Appendix A-2.

3. 12. 2. C Slickgone® NS

Slickgone® NS is Type 2/3 concentrate dispersants effective against a wide range of different oils including those with high wax contents. It is best applied undiluted but is compatible with sea water and can also be used diluted to 10%. Slickgone® NS is of low irritancy. Acute toxicity determined with Fish LC_{50} >1.8 g/L; invertebrate LC_{50} 0.393 g/L; and algae (*Skletonema costatin*) LC_{50} 1.8 g/L (Dasic International Ltd., 2007). The material, safety and data sheet is included in Appendix A-3.

3. 12. 3 Seawater

The natural seawater obtained by pumping from the near-shore wells, which provide sand filtration, was filtered through 0.45 µm Whatman® sterile membrane filter (Whatman® Limited, Maidstone England, Made in Japan) and used for the preparation water accommodated fraction and dilutions in bioassays. Water quality measurements of control/dilution water used in the test are reported in the (Table 3-3).

Table 3-3: Water quality measurements of seawater used in WAF and CE-WAF preparation

Parameter	Concentration
Dissolved Oxygen	10.0-13.4 mg/L
pH	7.0-8.16
Temperature	23.2-26.0ºC
Salinity	38.0-39.8 ppt
Conductivity	60.0-62.3 mS/s

3. 12. 4 Preparation of Kuwait Crude Oil WAF

There are several factors, which might individually and/or collectively affect the consistency of the prepared WAF solutions as mentioned previously such as mixing time, mixing speed, flask size and dimensions, pretreatment of dilution water by sterilization and/or filtration, etc. In the literature, preparation protocols have emerged after the conduction of several investigation attempts to understand the influences of each of the factors on the resulting prepared test medium. The preparation procedures described in the research reported in this study are based on the specific intention to generate an environmentally realistic test media intended to be used in toxicity testing which is if a highly repeatable composition. Through the estimation of the various chemical compounds' solubilities present in the WAFs solution, hydrocarbon concentrations can principally be determined. On the other hand, hydrocarbon concentrations in CE-WAFs are profoundly influenced by the presence of bulk oil droplets in the CE-WAFs solutions; because of physical processes (Singer et al., 2000).

In general, Singer et al., (2000) methodology was adopted to prepare WAF and CE-WAF of Kuwait crude oil form a single oil loading and subsequently making serial dilutions of the WAF and CE-WAF solutions for toxicity exposure. Hence, single oil loading of 1 g KCO/L filtered seawater (2 g KCO/2L seawater) was selected for the preparation of WAF and a 10:1 (oil:dispersant) ratio where 0.1 g oil dispersant (0.2 g oil dispersant/2L seawater) was selected and layered over the oil slick in a 2L glass aspirator bottle for the CE-WAF preparation. Crude oil and/or dispersants were layered over a known volume of filtered sea water, mixed for 24 h with 2.5/3.5 cm magnetic stir bar using a Bunsen® magnetic stirrer MC8 (Agitador Magnetic MCA), then stopped and the solution was left to stand for 3 h for a complete phase (oil/water) separation. WAF/CE-WAF solutions were drained, collected in amber bottles and preserved in a refrigerator until the moment of exposure.

Discussion of all dissolution experiments pertaining to Kuwait crude oil are summarized in the following section which discusses all of the four experiments in terms of the various parameters which most influence the overall consistency of WAF of Kuwait crude oil as follows:

- Effect of oil loadings
- Effect of mixing speed
- Effect of mixing time

3. 12. 5 Effect of Oil Loadings

The amount of test substance per unit volume of test medium is referred to as the loading rate (Girling et al., 1992). Increasing the test substance (crude oil)-to-medium (seawater) ratio will certainly increase the ability of substance's constituents to enter into the solution in the aqueous phase up to the limit of their aqueous solubility, if the differential partitioning effects of product constituents between the substance and the aqueous phase are disregarded. Large loading rates of test substance such as crude oil can provide a helpful basis for expressing and understanding the results of toxicity tests with products which are considered poorly soluble in seawater; as the amount of any substance with low solubility that is spilled in the marine environment will not be proportional to its water-solubility (Calow, 1998). It is a standard practice to produce a

range of dilution concentrations for acute toxicity testing after preparing a stock solution and then making serial dilutions to give a selected range of toxicity test solutions, which later LC_{50} concentrations can be calculated from. When making a series of WAF preparations, one might expect that the least water-soluble components in the oil may attain their maximum concentration in the lowest oil-loading rate used and this concentration will not change as the oil loading rate change. The composition of the aqueous phase can be subjected to various changes at individual oil loading. Therefore, when dealing with complex mixtures of poorly soluble components such as crude oil it is imperative to specify the oil-loading rate of the test material to dilution water and not to completely rely on the initial prepared stock solution where all present materials will remain in a similar ratio to one another (CONCAWE, 1992f).

3. 12. 6 Effect of Mixing Speed

There are various ways that mixing of WAF solution can be performed, that include (swirling, shaking, rotation, etc.) in which a magnetic stirrer bars can be used for any vessel size which are placed on a Bunsen® stirrer plate. Electromagnetic stirrers provide a high level of accuracy and repeatability because they do not consist of any additional moving parts that might be vulnerable to deterioration over time with continuous use. Fundamentally, in this research study for the preparation of WAF and CE-WAF, mixing speeds were selected to sufficiently generate a 20-25% vortex in the water column and these mixing speeds were (300 and 650 rpm) for a mixing period of 24 and 120 h. All other conditions are left constant such as: vessel size, flushing method, mixing time, etc.

3. 12. 7 Effect of Mixing Time

Mixing time is considered as an essential parameter during the preparation of crude oil WAF in order to achieve a substantial level of "functional equilibrium" within the preparation flask. In other words, the aqueous phase resulting from mixing the crude oil with seawater should be saturated in terms of soluble compounds present that are petroleum hydrocarbons (Singer et al., 2000). In this research, a comparison was conducted between: (a) difference between 24 and 120 h of mixing using 5 g KCO/l seawater (10 g KCO/2L seawater) for WAF preparation, and (b) several mixing

duration such as (30 min, 1, 2, 4, 8, 24, and 48 h) using 1 g KCO/L seawater (2 g KCO/2L seawater) for WAF preparation. All other conditions were kept constant such as: flushing method, mixing energy, mixing duration, and vessel type (aspirator bottle) as described in (Table 3-4).

Table 3-4: Experimental conditions used for the effect of various mixing time on BTEX and TPH concentration experiment

Crude Oil (g)	Seawater Volume (L)	Mixing Time (h)	Mixing Speed (rpm)	Settling Time (h)	Stirrer Bar Size (cm)	Vessel Size (L)	Vortex	Flushing Method
5	1 L	24 and 120	300	3	2.5	2	25% of the water column	Gravity
1	1 L	30 min, 1, 2, 4, 8, 24, and 48	300	3	2.5	2	25% of the water column	Gravity

3. 12. 8 Flushing Method

Gravity method was selected, from various experimental methodologies in published literature (Saeed et al., 2000), where WAF solution was drained from the mixing vessel nozzle by a method of gravity and collected in another collection flask. Moreover, an attempt was made to observe how the crude oil selected in this study behaves with the gravity flushing method. A 5 g KCO/L seawater was used to prepare WAF solution as gravity was used to drain the mixing solution.

3. 12. 9 Preparation of WAF Experiments

Various WAF preparation methods were tested for the main purpose of producing a chemical mixture, which is toxicologically reproducible when tested on different marine species. There are several factors that collectively influence the overall composition of WAF and are considered highly significant during the process of WAF preparation. Such factors are mixing speed, mixing time, crude oil-to- seawater ratio,

pretreatment of dilution water (seawater) through sterilization, and WAF collection method. Moreover, effects of flask dimensions, and separation time, etc. can affect the consistency of WAF chemical composition.

In this study, test conditions and variables used in the preparation of KCO WAF solution are given in (Table 3-5, Figure 3-1 and 3-2). Four experiments were conducted by varying one or the other parameter as discussed below:

3. 12. 9. A Experiment No. 1

Variable oil loadings of KCO WAF were prepared using five oil loadings 0.1, 0.5, 1.0, 3.0, and 10 g KCO/L seawater (0.2, 1.0, 2.0, 6.0 and 20.0 g KCO/2L seawater) as indicated in (Table 3-5) and all other conditions kept constant and WAF was collected by gravity method.

3. 12. 9. B Experiment No. 2

KCO WAF was prepared using single oil loading of 5 g KCO/L seawater (10 g KCO/2L seawater) (Table 3-5). The variables in this experiment were two stirring rates (300 and 650 rpm) and two stirring durations (24 and 120 h). Gravity was used as a flushing method to drain the prepared WAF.

3. 12. 9. C Experiment No. 3

In this experiment lower oil-to-seawater loading 1 g KCO/L seawater (2 g KCO/2L seawater) was used and the effect of mixing duration was examined. The mixing was done for these selected time periods (30 min, 1, 2, 4, 8, 24, and 48 h). Gravity was used as a flushing method to drain the prepared WAF and other conditions used were the same as indicated in (Table 3-5).

3. 12. 9. D Experiment No. 4

This experiment had two integral parts which were investigated: 1) the effect of KCO WAF and CE-WAF on BTEX and TPH concentrations and 2) the effect of preparation method by: (a) delivery of chemical into the vortex (DIV) and (b) delivery

of chemical not into the vortex (DNIV) on BTEX and TPH concentrations. The details of experimental settings were:

1) The ratio loading ratio was 0.1 g/1 g/1L dispersant/KCO/seawater (0.2 g/2 g/2L dispersant/KCO/seawater), for three oil dispersants Corexit EC® 9500, Corexit EC® 9527, and Slickgone® NS; respectively. The fourth chemical treatment, water-accommodated fraction of Kuwait crude oil alone (KCO WAF) was prepared using single oil loading which was 1 g KCO/L seawater ratio. All other preparation conditions were kept the same except that stirrer bar was changed to 3.5 cm for a mixing period of 24 h to examine the effect of increased magnetic bar size on BTEX and TPH concentrations. The solution was left to stand for 3 h for a complete phase separation (oil and water) after which it was drained by gravity method (Table 3-5).

2) In addition, Singer et al., (2000) compared two CE-WAF preparation methods in which firstly, oil was layered over a still mass of seawater then mixing was initiated until a vortex was established; after that a dispersant was added to the swirling mass. Secondly, an alternative method was used in which a vortex was initiated in the seawater mass first, and then oil plus dispersants were both added into the vortex center sequentially. Therefore, an investigation of chemical delivery method was conducted in which two preparation conditions were used: 1) delivery of test chemical into vortex (DIV), and 2) delivery of test chemical not into the vortex (DNIV). All test chemicals prepared were subjected to BTEX, TPH, and PAHs analysis and the WAF and CE-WAF chemical mixtures were prepared in four replicates (Table 3-5).

Table 3-5: Test conditions for dissolution of Kuwait crude oil in seawater experiments

Test Conditions for Dissolution of Kuwait Crude Oil in Seawater Experiments				
	Number of Experiments			
Conditions	**1**	**2**	**3**	**4**
Seawater	Shore wells seawater filtered with 0.45 µm filter paper	Shore wells seawater filtered with 0.45µm filter paper	Shore wells seawater filtered with 0.45 µm filter paper	Shore wells seawater filtered with 0.45 µm filter paper
Vessel	2 L glass aspirator bottle	2 L glass aspirator bottle	2 L glass aspirator bottle	2 L glass aspirator bottle
Volume of Seawater	2L	2 L	2 L	2 L
Volume of Crude Oil	0.1, 0.5, 1.0, 3.0, and 10.0 g	5 g	2 g	2 g
Volume of Oil Dispersant	-	-	-	0.2 g
Head Space	25% of beaker size	25% of beaker size	25% of beaker size	25% of beaker size
Stirrer Bar Size	2.5 cm	2.5 cm	2.5 cm	3.5 cm
Mixing Speed	300 rpm	300 and 650 rpm	300 rpm	300 rpm
Mixing Time	24 h	24 h and 120 h	30 min, 1, 2, 4, 8,16, 24, and 48 h	24 h
Settling Duration	3 h	3 h	3 h	3 h
Vortex	5% of water column	5% of water column	5% of water column	5% of water column
WAF Flushing Method	Gravity	Gravity	Gravity	Gravity
Test Temperature	20°C	20°C	20°C	20°C

Figure 3-1: Preparation of Kuwait crude oil WAF and CE-WAF solutions in laboratory

3. 12. 10 Chemical Characterization of KCO WAF and CE-WAF Solutions

WAF and CE-WAF of Kuwait crude oil and Kuwait crude oil plus oil dispersants were characterized for the presences of the following compounds: BTEX, TPH, individual PAHs and aliphatic compounds for selected preparations according to the methods indicated in (Table 3-6). To date, various analytical data related to WAF of crude oil and CE-WAF of dispersed crude oil has been generated to standardize analytical methods. Unfortunately, recognizable amounts of these data are not comparable because of vast differences in analytical and toxicological methods implemented in addition to lack of analytical verification exposures. Therefore, both analytical and toxicological method needed to be developed to produce acute toxicity data relevant to native marine species associated with Kuwait's marine ecosystem which represents different hierarchical and tropic levels. The toxicity data are in need for further development which are associated with complex chemical mixtures such as Kuwait crude oil, specific chemical dispersants and dispersed Kuwait crude oil.

Table 3-6: Chemical characterization of KCO WAF and CE-WAF solutions

	Test Chemical	Analysis	Methods
1.	KCO WAF	TPH	Fluorometric Analysis
		BTEX: Benzene, Toluene, Ethylbenzene, Xylenes	GC/MS
		PAHs	GC/MS
2.	CE-WAFs		
		TPH	
	Corexit® 9500 + KCO	BTEX:Benzene,Toluene,Ethylbenzene, Xylenes,	Fluorometric Analysis
		Individual PAHs	
	Corexit® 9527 + KCO		GC/MS
	Slickgone® NS + KCO		GC/MS

3. 12. 11 BTEX Analysis

BTEX compounds were directly estimated in WAF and CE-WAF of Kuwait crude oil using EPA METHOD 5035 (Closed-system purge-and-trap extraction for volatile organics in soil and waste samples) (1996). Standard BTEX stock concentration of each analyte (benzene, toluene, ethyl benzene and xylene) was prepared from stock solution 200 mg/L to 4.0 ng/mL. A stock solution of 200 mg/L of all BTEX was prepared to 2 mg/L after taking 0.1 mL to 1.0 mL methanol (CH_3OH), then again from the 2 mg/L, 10 mL diluted to 5 mL (VOC) free distilled water (4 ng/mL). The sample concentration was prepared by diluting the sample after taking 0.01 mL and diluting it to 5.0 mL VOC free distilled water. After setting all the method conditions, 5 mL of standard solution (4.0 ng/mL) was injected in purge and trap system using glass syringe. After calibration 5 mL of blank was injected to make sure that the system is free from any impurities, and then the diluted 5 ml sample was injected with the 5 mL glass syringe. BTEX compounds were determined by GC/MS with purge and trap method. Instrumental settings are indicated in (Table 3-7).

Table 3-7: GC/MS with purge and trap instrumental settings

GC/MS with Purge and Trap Instrumental Settings	
Standard	UST BTEX Standard Catalog No. 48026/4S8026 This mixture contains 200µg/mL of each of the BTEX components in Methanol
Trap	VOCARB 3000
Purge	11 min 40 mL/min
Dry	3 min
Desorbtion Temperature	250 °C for 4 min
Bake	280 °C for 10 min
Column	DB 627 X 0.32 ID X 30 m.
Oven	35°C (6 min) to 180°C at 5.50°C/min, hold 9 min to 220°C at 9.50°C/min, hold 2 min
Carrier	Helium, 1.3 mL/min.
Detection	MS, Scan Range m/z = 35-260at 0.6 sec/scan.
BTEX Detection Limit	< 0.001 mg/L
HP Model	Agilent Technologies 6890N Network GC System. 5973 inert Mass Selective Detector

3. 12. 12 Total Petroleum Hydrocarbon (TPH) Analysis

Many of the experimental parameters can affect the extraction of WAF and/or WSF from crude oil such as: crude oil type, ratio of oil to seawater, energy and duration of mixing, settling time necessary to achieve a constant distribution of hydrocarbon compounds between the aqueous and oil phases (Zhou et al., 1994). TPH were extracted from 100 ml WAF and CE-WAF by initially adding 20 mL of MERCK® dichloromethane (CH_2Cl_2) in clean and solvent rinsed 2L separatory funnel with Teflon stopcock and stopper. The mixture was shaken vigorously for 10 s, the cap was loosened and the solvent was allowed to escape as pressure was building up because of shaking the mixture and the separating funnel was racked and the solvent phase was allowed to separate. This step was repeated three times then the medium was dried over a few grams of MERCK® grade anhydrous sodium sulfate (Na_2SO_4) and glass wool, which were presoaked and rinsed with dichloromethane. This step was repeated twice by adding another 40 mL (20 ml each time) then drying the resultant solution again over anhydrous sodium sulfate and glass wool then the solvent layer was withdrawn and collected in 100 mL volumetric flask which was labeled and stored for later analysis. The collected extract was then analyzed on a RF-5301 PC SHIMADZU® Spectrofluorophotometer instrument using 310 nm excitation and 360 nm emission wavelengths (Table 3-8). The levels of TPH were calculated against a prepared standard multipoint calibration curve using 10 mg of Kuwait crude oil and reported in terms of the KCO equivalents (MOOPAM, 1999). A standard calibration curve was generated for TPH analysis and (Table 3-9, Figure 3-2, 3-3 and 3-4).

Table 3-8: Shimadzu® spectrofluorophotometer instrumental settings

RF-5301 PC SHIMADZU® Spectrofluorophotometer Instrumental Settings	
Method	Quantitative
Concentration	µg/mL
Range	0.000-50.000
Recording Range	Low: 0.000- High: 100
Method	Multi point working curve
Slit Width	EX: 3- EM: 3
Excitation Wavelength	310 nm
Emission Wavelength	360 nm
Recorded Range	0.00-1000
Sensitivity	High
Response Time	Auto
Repetitions	5

Table 3-9: Multi-point standard calibration curve using 10 mg of Kuwait crude oil

Concentration (mg/L)	**Fluorescence**
0	0.472
0.1	23.808
0.2	28.144
0.5	34.042
1.0	43.218
2.0	62.113
5.0	127.593
10.0	219.474
20.0	355.242

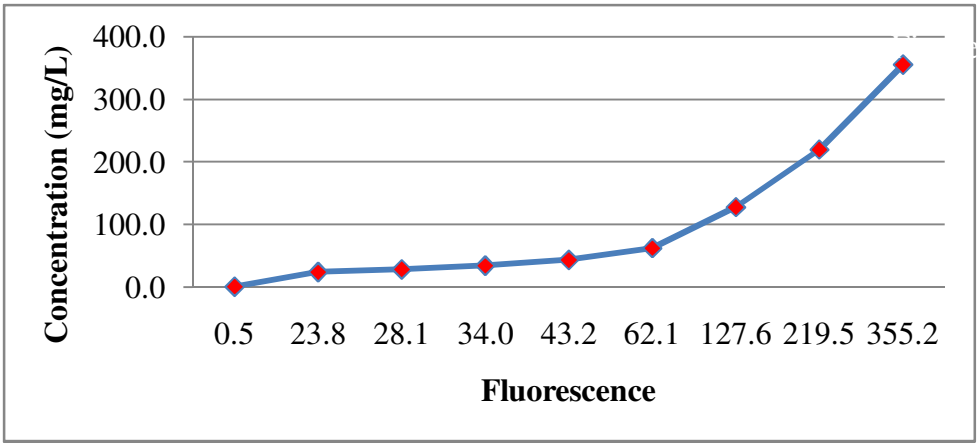


Figure 3-2: Standard multi-point calibration curve generated by Shimadzu® Spectrofluorophotometer using 10 mg of Kuwait crude oil

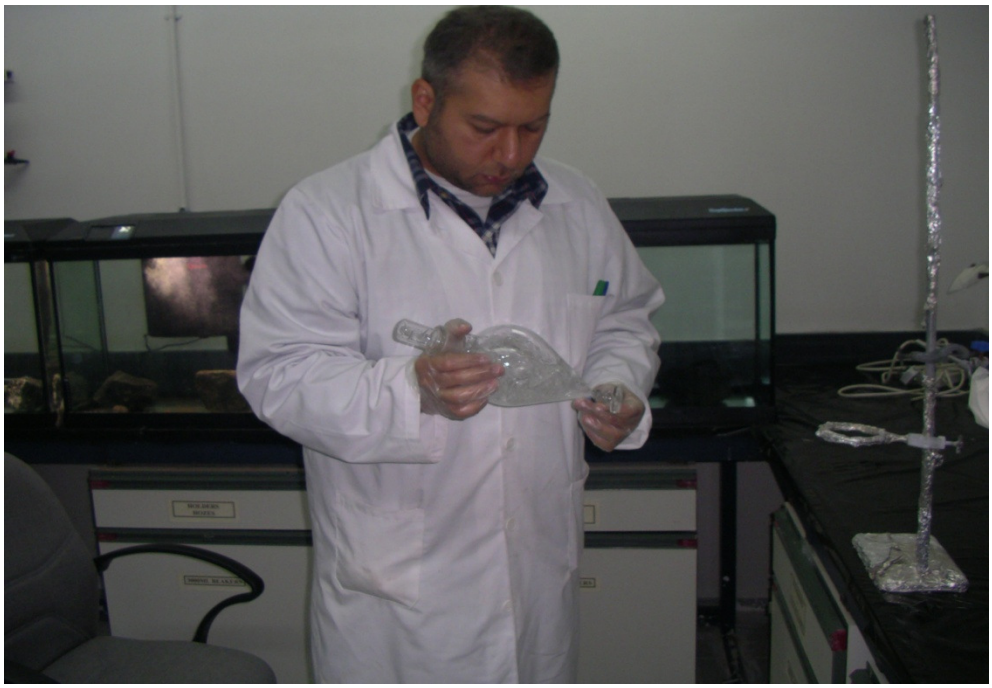


Figure 3-3: Chemcial extraction of Kuwait crude oil WAF and CE-WAF with dichloromethane and collection of extracted solution

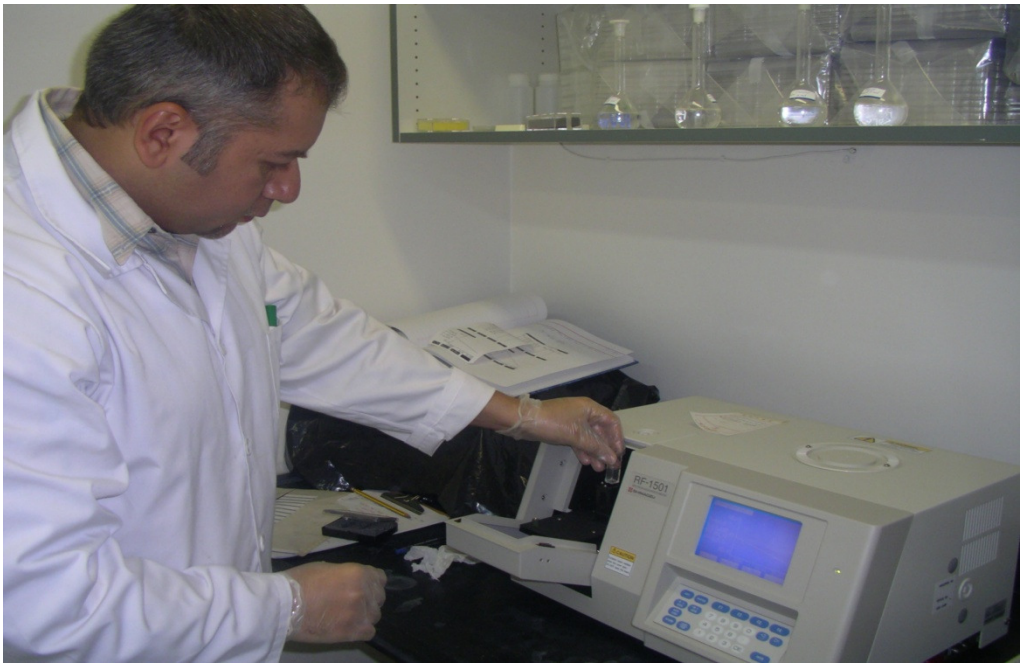


Figure 3-4: TPH Analysis of WAF and CE-WAF solutions using Shimadzu® Spectrofluorophotometer instrument

3. 12. 13 Total Polycyclic Aromatic Hydrocarbons (PAHs) Analysis and Aliphatics

Polycyclic Aromatic Hydrocarbons were analyzed according to Bondi et al., (2006) adopted for seawater samples. Dichloromethane extracts used for total PAH determination were cleaned and reduced for the estimation of individual PAHs by the GC-MS method. The individual PAHs were analyzed using a Shimadzu® GC-17A gas chromatograph using split-less injection on a 30 m HP5-ms column (0.25 mm i.d., 0.25µm film thickness) and helium as the carrier gas. This was coupled to a Shimadzu® QP-5050A mass selective detector operated in electron impact (EI) mode using selected ion monitoring (SIM) as given in (Table 3-10). Identification and quantification was carried out against 5 calibration standards of known concentration. A peak was positively identified if it was within ±0.05 min of the retention time in the calibration standard and quantified only if the S/N=3 and the ratio of the ion to its qualifier ion were within ±20% of the standard value. The PAHs standarads used were: PAHs Mixture HP Part # 8500-6035 and internal and surrogates STD Fortification Solution HP Part # 8500-6076. The list of individual PAHs analyzed and ions monitored are indicated in (Table 3-11). For Aliphatics, an elution was perfomed using 3 ml hexanto yield the fraction which contains the aliphatic hydrocarbons then injected into GC-MS instrument according to (MOOPAM, 1999).

Table 3-10: GC Instrumental settings

GC Instrumental Settings	
Injector Temperature	290 ^{o}C
Transfer Line	280 ^{o}C
Initial Oven Temperature	60 ^{o}C
Initial Hold	1 min
Ramp Rate	6 ^{o}C/min
Final Temp	280 ^{o}C
Flow Rate	20 min
Carrier Gas	Helium
Final Hold	1.7 mL/min

Table 3-11: List of individual PAHs analyzed and ions monitored

Compound Name	Ions	
d_8-Naphthalene	136	137
Naphthalene	127	128
d_8-Acenaphthylene	159	160
Acenaphthylene	151	152
Acenaphthene	153	154
Fluorene	165	166
Phenanthrene	178	179
d_{10}-Anthracene	188	189
Anthracene	178	179
d_{10}-Fluoranthene	212	213
Fluoranthene	200	202
d_{10}-Pyrene	212	213
Pyrene	200	202
9-butyl phenanthrene	191	234
Benz[a]anthracene	226	228
Chrysene	226	228
Benzo[b]fluoranthene	252	253
Benzo[k]fluoranthene	252	253
Benzo[a]pyrene	252	253
Indo[123,cd]pyrene	274	276
Dibenzo[ah]anthracene	276	278
d_{12}-Benzo[g,h,i]perylene	288	289
Benzo[g,h,i]perylene	274	276

3. 12. 14 TPH Concentration Change in Serial Dilutions of KCO WAF Experiment

Since serial dilutions of KCO WAF solutions are normally used for conducting a 96 h acute toxicity bioassay, it was essential to understand the behavior of KCO WAF and CE-WAF in the exposure system (100 mL beakers) and determine the change of total petroleum hydrocarbons (TPH) concentrations during the 96 h exposure period in abiotic conditions that is without the presence of any organisms. A 50 mL of KCO WAF was transferred to a 100 mL glass beaker which made the 100% dilution. The subsequent five-dilution beakers had 50 mL of filtered seawater in which 50 mL of the 100% beaker was added and serial dilutions of KCO WAF mixed with filtered seawater were made until the last sixth beaker. Serial concentration dilutions are prepared in this fashion (3.12, 6.25, 12.5, 25, 50 and 100%). Exposure beakers were covered with Parafilm® to eliminate any loss of solution and further external contamination. KCO WAFs (50 mL) were extracted with (60 mL) dichloromethane and TPH concentrations were determined using a spectrofluorophotometer.

3. 12. 15 Fourier Transform Infra Red Spectroscopy (FT-IR) Analysis for TPH

FT-IR analysis was used to investigate the composition of the KCO WAF and CE-WAFs analyzed in terms of TPH concentration. KISR/CAL-S01/95 modified method from EPA method 418.1 (1978) was used to analyze the prepared samples. WAF and CE-WAF samples were extracted with CCl_4 (300 mL of water in 50 mL of CCl_4) and the TPH in the collected extracts were quantified by FT-IR instrument. The measurable detection limit at optimized conditions was at 0.2 mg/L. The extract was filled up to 100 mL and the IR absorbance spectrum of the extract was recorded in the range from 3500 cm/L to 2500 cm/L using a rectangular quartz cell. The area under the signal of the aromatic and aliphatic CH valence vibration (3150 cm/L to 2750 cm/L) was measured. The integration value was converted into the concentration value by means of a calibration curve. A calibration curve standard was prepared by making a stock solution of 15 mL n-tetradecane, 15 mL iso-octane, and 10 mL benzene. Calibration points from 1-40 mg/L were selected to make the calibration curve as indicated in (Table 3-12) for instance 0.1 mL from stock solution was pipetted into a 100 mL volumetric flask and then diluted to 100 mL with CCl_4.

Table 3-12: Integral values for calibration standards in CCl_4 using a 50 mm cell

Concentration (mg/L)	Integral 3150 to 2750 (cm/L)
1	2.9
2	3.9
4	5.0
6	9.2
8	8.0
10	11.2
12	12.2
16	15.9
20	20.6
24	24.0
28	27.4
30	32.2
32	31.8
36	35.6
40	38.7

3. 12. 16 Direct Fluorescence Measurement of WAF and CE-WAF

The excitation and emission wavelengths used for TPH measurements were used for direct fluorescence measurements of WAF and CEWAF solutions to determine the change in fluorescence during the 96 h acute bioassay without the addition of any organisms (abiotic conditions).

WAF samples were not extracted with dichloromethane, only a direct measurement of fluorescence was performed in RF-5301 PC SHIMADZU® Spectrofluorophotometer with increased slit width (5/5) and high sensitivity. Percent loss of KCO WAF and CE-WAF solutions was determined to using the following equations:

$$\% \text{ Loss} = \frac{(0 \text{ h absorbance} - 24 \text{ h absorbance}) \times 100}{0 \text{ h absorbance}}$$

3. 12. 17 Ultraviolet UV Spectrophotometry Analysis

The WAF, CE-WAF, and/or WSF can be quantified by measuring absorbance at a specifically selected wavelength, which can be compared with the absorbance of extracts prepared from arbitrary oil standards. The selected wavelength is chosen on the basis that it should give the maximum absorption when a chemical mixture sample is analyzed. In this study SHIMADZU® UV-1601 UV-VISIBLE Spectrophotometer was used to directly analyze WAF and CE-WAF samples prepared in four replicates at a selected wavelength of 253 nm. Results of all analyses are described in (Table 3-13).

3. 13 Results

Table 3-13: Influence of preparation conditions on BTEX and TPH concentrations of KCO WAF and CE-WAF

			BTEX Concentration (mg/L)				
Expt. No.	**Test Variables**	**Test Conditions**	**Benzene**	**Toluene**	**Ethylbenzene**	**Xylene**	**TPH (mg/L)**
1	KCO WAF Oil loadings: 0.1, 0.5, 1.0, 3.0, and 10.0 g KCO/L seawater (SW), 2.5 cm bar stirrer size.	0.1 g KCO/L SW	0.02	0.007	0.03	0.04	0.091
		0.5 g KCO/L SW	0.6	1.7	0.9	0.2	0.221
		1.0 g KCO/L SW	1.1	2.8	0.5	0.6	0.231
		3.0 g KCO/L SW	1.9	2	0.4	0.5	0.342
		10.0 g KCO/L SW	1.4	2	0.3	0.3	0.682
2	KCO WAF Oil loading: 5 g KCO/l SW, mixing speed: 300 & 650 rpm, mixing times: 24 and 120 h, 2.5 cm stirrer bar size.	(300 rpm, 24 h)	0.49	0.663	0.083	0.173	0.11
		(300 rpm, 120 h)	1.066	1.773	0.273	0.323	0.12
		(650 rpm, 24 h)	0.527	0.873	0.09	0.18	0.10
		(650 rpm, 120 h)	0.563	0.973	0.147	0.187	0.11
3	KCO WAF Oil loading: 1 g KCO/L SW mixing times: 30 min, 1, 2, 4, 8, 24 and 48 h, 2.5 cm stirrer bar size.	After 30min.	0.008	0.038	0.002	0.001	0.08
		After 1 h	0.11	0.2	0.02	0.03	0.06
		After 2 h	0.12	0.25	0.02	0.03	0.07
		After 4 h	0.24	0.62	0.08	0.09	0.28
		After 8 h	0.35	0.14	0.12	0.19	0.19
		After 24 h	0.41	1.13	0.22	0.27	0.28
		After 48 h	0.15	0.73	0.08	0.13	0.28
4	Oil/ Dispersant ratio: 1 g KCO + 0.1 g dispersant/L SW, 3.5 cm stirrer bar size	KCO WAF	0.403	0.119	0.247	0.307	0.5
		Corexit® 9500 CE-WAF	0.3467	1.0033	0.1833	0.2333	23.7
		Corexit® 9527 CE-WAF	0.51	1.5733	0.3467	0.43	35.2
		Slickgone® NS CE-WAF	0.5667	1.6167	0.3033	0.39	3.0
	1) Delivery into the vortex (DIV)	KCO WAF	0.405	1.178	0.24	0.298	0.1
		Corexit® 9500 CE-WAF	0.38	1.088	0.278	0.263	16.5
		Corexit ®9527 CE-WAF	0.445	1.348	0.305	0.373	17.2
		Slickgone® NS CE-WAF	0.567	1.617	0.303	0.39	7.2
	2) Delivery NOT into the vortex (DNIV)	KCO WAF	0.317	1.023	0.18	0.22	0.48
		Corexit® 9500 CE-WAF	0.507	1.233	0.27	0.33	23.7
		Corexit® 9527 CE-WAF	0.34	1.3	0.4333	0.32	35.2
		Slickgone® NS CE-WAF	0.34	1.167	0.257	0.307	3.04

3. 13. 1 Experiment No. 1

The effect of five oil loadings on BTEX and TPH concentrations in KCO WAF as indicated in Table 3-14 varied and the concentration of BTEX was not consistent with oil loadings. In the 0.1 g KCO loading, xylene was the compound with the highest concentration (0.04 mg/L) followed by ethylbenzene (0.03 mg/L), then benzene (0.02 mg/L) and finally toluene (0.007 mg/L). At 0.5 g KCO loading and above, toluene was the highest among BTEX compounds. Benzene was present in general at second highest concentration and ethylbenzene and xylene fluctuated in similar range. At 1.0 g KCO loading, except for benzene; other compounds were higher in WAF compared to higher oil loadings at 3.0 and 10.0 g KCO. The BTEX were not further enriched in WAF by increasing oil loadings from 1.0 g to 3.0 or 10.0 g KCO/L seawater. On the other hand, TPH concentrations increased in WAF with the increasing oil loadings. It increased from (0.019 mg/L) for 0.1 g KCO/L seawater loading until it reached (0.682 mg/L) for 10.0 g KCO/L seawater loading.

Table 3-14: Average BTEX and TPH concentrations in KCO WAF at various oil loadings (Exp. 1)

BTEX Compounds Concentration (mg/L)					TPH (mg/L)
KCO (g) + Seawater (L)	**Benzene**	**Toluene**	**Ethylbenzene**	**Xylene**	
0.1 g KCO + l SW	0.02	0.007	0.03	0.04	0.019
0.5 g KCO + l SW	0.6	1.7	0.9	0.2	0.221
1.0 g KCO + l SW	1.1	2.8	0.5	0.6	0.231
3.0 g KCO + l SW	1.9	2	0.4	0.5	0.342
10.0 g KCO + l SW	1.4	2	0.3	0.3	0.682

3. 13. 2 Experiment No. 2

The effect of two mixing speeds (300 and 650 rpm) and two mixing times (24 and 120 h) on BTEX and TPH concentrations are indicated in Table 3-15. The data showed that at low mixing speed, (300 rpm) WAF had less BTEX concentrations in the 24 h mixing time, and increasing the mixing time to 120 h almost doubled the BTEX concentration in WAF but the pattern of each of the BTEX compounds remained the same. Whereas at high mixing speed BTEX concentrations remained more or less constant with minor increases in long term preparations. TPH concentrations were not affected in either case. The data at higher mixing speed and 24 h mixing time resulted in slightly higher BTEX concentrations compared to that found in WAF prepared at the low mixing speed for the same time. Increasing mixing time at high speed brought no change in BTEX concentration found at 24 h, except that ethylbenzene concentration was appreciably higher in WAF prepared by long mixing times. TPH concentration was approximately the same in WAF prepared at higher speed at 24 and 120 h mixing times.

Table 3-15: Average BTEX and TPH concentrations at various mixing times and mixing speeds (Exp. 2)

Experiment Conditions	KCO (g) + seawater (SW)	BTEX Compounds Concentration (mg/L) Benzene	Toluene	Ethylbenzene	Xylene	TPH (mg/L)
(300 rpm and 24 h)	5 g KCO + 1 SW	0.49	0.663	0.083	0.173	0.111
(300 rpm and 120 h)	5 g KCO + 1 SW	1.066	1.773	0.273	0.323	0.117
(650 rpm and 24 h)	5 g KCO + 1 SW	0.527	0.873	0.09	0.18	0.103
(650 rpm and 120 h)	5 g KCO + 1 SW	0.563	0.973	0.147	0.187	0.106

3. 13. 3 Experiment No. 3

Lower oil loadings (1 g KCO/L seawater) were also used for WAF preparation at several mixing duration such as (30 min, 1, 2, 4, 8, 24, and 48 h) (Table 3-16). The effect of several mixing times (30 min, 1, 2, 4, 8, 24, and 48 h) using 1 g KCO/L seawater for KCO WAF preparation. The data for the WAF prepared at lower oil loading (1 g KCO/L seawater) and low mixing speed (300 rpm) with various mixing times from 30 min to 48 h are reported in Table 3-16.

Amongst the four BTEX compounds (benzene, toluene, ethylbenzene and xylene), toluene produced the highest concentrations up to 24 h (1.13 mg/L) then declined at 48 h (Table 3-16). Next was benzene, which resulted in a lower concentration than toluene, but was second highest in concentration among the BTEX compounds. However, the pattern was the same with all BTEX compounds which demonstrated a gradual increase starting from the first 30 min up until the 24 h of mixing duration then a decline was observed.

TPH concentrations demonstrated a linear relationship and a consistent pattern with the selected mixing durations. TPH increased from (0.08 mg/L) for the first 30 min of mixing duration until it reached (0.28 mg/L) for the 24 and 48 h of mixing (Table 3-16). TPH concentration stabilized within the 24 and 48 h of mixing duration and reached saturation level at this time intervals. Unlike BTEX results, TPH values produced a more prominent and reliable understanding of how the selected oil loading of 1 g KCO/L seawater loading behaved when exposed to various mixing durations until reaching saturation point. Thus, concluding that 24 h time interval is the most appropriate mixing duration to produce a WAF solution intended to be used for toxicity testing. The selected 1 g KCO/L seawater loading behaved more systematically than 5

g KCO/L seawater loading, and the TPH concentrations were slightly different between the 1 and 5 g KCO volumes used to prepare the WAF solution. TPH concentration (0.28 mg/L) was with two orders of magnitude more than the one produced from using 5 g KCO WAF (0.11 mg/L).

Table 3-16: Average BTEX and TPH concentration of KCO WAF at various mixing times (Exp. 3)

			BTEX Compounds Concentration (mg/L)				
Sample No.	**Crude Oil (g KCO/L seawater**	**WAF Collection Time (min & h)**	**Benzene**	**Toluene**	**Ethylbenzene**	**Xylene**	**TPH (mg/L)**
1		After 30 min	0.008	0.038	0.002	0.001	0.08
2		After 1 h	0.11	0.2	0.02	0.03	0.06
3		After 2 h	0.12	0.25	0.02	0.03	0.07
4	1 g/L	After 4 h	0.24	0.62	0.08	0.09	0.28
5		After 8 h	0.35	0.14	0.12	0.19	0.19
7		After 24 h	0.41	1.13	0.01	0.01	0.28
8		After 48h	0.15	0.73	0.08	0.13	0.28

3. 13. 4 Experiment No. 4

WAF of Kuwait crude oil and CE-WAF of Kuwait crude oil plus three individual oil dispersants have been analyzed for BTEX and TPH as indicated in Table 3-17. The four WAF and CE-WAF chemical mixtures produced various BTEX concentrations. Slickgone® NS CE-WAF had the highest benzene concentration among the four chemical mixtures which was (0.566 mg/L) followed by Corexit® 9527 CE-WAF (510.0 mg/L), then KCO WAF (0.4033 mg/L) then finally, Corexit® 9500 CE-WAF (0.346 mg/L) as the CE-WAF with the lowest benzene concentration. As for the second BTEX compound toluene, it followed the same pattern as that found with benzene where Slickgone® NS CE-WAF had the highest toluene concentration among the four chemical mixtures prepared. Ethyl benzene concentration was the highest in Corexit® 9527 CE-WAF followed by Slickgone® NS CE-WAF, KCO WAF, and finally Corexit® 9500® CE-WAF. Xylene was the highest in Corexit® 9527 CE-WAF followed by Slickgone® NS CE-WAF, KCO WAF and finally Corexit® 9500 CE-WAF. TPH concentrations (Table 3-17) varied in the four chemical preparations (WAF and CE-WAFs) in a sense that CE-WAF of Corexit® 9527 had the highest TPH concentration,

followed by Corexit® 9500 CE-WAF, then Slickgone® NS CE-WAF, and finally KCO WAF.

The investigation of the effect of two preparation conditions that is: delivery of test chemical into the seawater vortex (DIV) and delivery of test chemical not into the seawater vortex (DNIV) yielded different results in terms of BTEX compounds as indicated previously in Table 3-13. The DIV preparation method for KCO WAF resulted in slightly higher concentrations of some of the BTEX compounds than in the DNIV method, but it resulted in lower TPH concentration. As for Corexit® 9500 CE-WAF, the DNIV preparation method resulted in higher benzene, toluene, ethylbenzene, and TPH concentrations than the DIV method, but it resulted in similar xylene concentration to the DIV method. For Corexit® 9527 CE-WAF, the DIV preparation method resulted in higher benzene and xylene concentrations than DNIV method, but resulted in lower ethylbenezene and TPH concentration; toluene concentration was the same for both methods. Finally, for Slickgone® NS CE-WAF; the DIV method resulted in higher BTEX and TPH concentrations than the DNIV method. When the effect of stirrer bar size was examined (Table 3-13), it indicated that using 2.5 cm bar size resulted in higher BTEX concentrations compared to what was obtained after using 3.5 cm bar size. For instance, toluene concentration (2.8 mg/L) for 2.5 cm bar size was the highest among BTEX compound compared to 0.119 mg/L for 3.5 cm bar size. In general, after using 2.5 cm bar size; other BTEX compounds had a concentration nearly double than what was obtained after using 3.5 cm bar size. TPH concentration for 2.5 cm bar size (0.231 mg/L) was slightly less than that of 3.5 cm bar size (0.5 mg/L) and the difference was not significant.

Table 3-17: Average BTEX and TPH concentrations (mg/L) for KCO WAF and CE-WAF (Exp. 4)

	BTEX Compounds Concentration (mg/L)				TPH
Chemical Name	**Benzene**	**Toluene**	**Ethylbenzene**	**Xylene**	**(mg/L)**
KCO WAF	0.4033	1.1933	0.247	0.307	0.5
Corexit® 9500 CE-WAF	0.3467	1.0033	0.1833	0.2333	23.7
Corexit® 9527 CE-WAF	0.51	1.5733	0.3467	0.43	35.2
Slickgone® NS CE-WAF	0.5667	1.6167	0.3033	0.39	3.0

3. 14 Individual PAHs in WAF and CE-WAF

Individual polycyclic aromatic hydrocarbons were determined in KCO WAF and CE-WAF (Table 3-18). For KCO WAF, among sixteen PAH compounds determined; the following PAHs were present such as: naphthalene (21.732 ng/mL), phenanthrene (0.409 ng/mL), fluorene (0.379 ng/mL), fluoranthrene (0.024 ng/mL), and pyrene (0.011 ng/mL). PAHS with high molecular weights were mostly not detected. Except Corexit® 9500 treated CE-WAF had total individual PAHs which were lower in CE-WAF compared to KCO WAF. In Corexit® treated CE-WAFs; chrysene was the additional compound that was detected which was not present in either KCO WAF or Slickgone® NS CE-WAF treatment. Corexit® 9500 CE-WAF had the highest total PAH concentration among all test chemicals > KCO WAF > Corexit® 9527 CE-WAF > Slickgone® NS CE-WAF.

Table 3-18: Individual PAHs in KCO WAF and CE-WAF

Individual PAHs Concentrations (ng/mL)					
		WAF and CE-WAF Concentrations			
		1 g KCO/L Seawater	**1 g KCO/0.001 g Dispersant/L Seawater**	**1 g KCO/0.001 g Dispersant/L Seawater**	**1 g KCO/0.001 g Dispersant/L Seawater**
No.	**Compound Name**	**KCO WAF**	**Corexit® 9500 CE-WAF**	**Corexit® 9527 CE-WAF**	**Slickgone®NS CE-WAF**
1	Naphthalene	21.732	21.542	12.859	16.04
2	Acenaphthylene	0.089	0.485	0.547	n.d.
3	Acenaphthene	0.127	n.d.	n.d.	n.d.
4	Fluorene	0.379	1.645	1.808	0.340
5	Phenanthrene	0.409	2.658	2.440	0.419
6	Anthracene	n.d.	n.d.	n.d.	n.d.
7	Fluoranthene	0.024	0.443	0.093	0.034
8	Pyrene	0.011	n.d.	0.168	0.017
9	Benzo(a)anthracene	n.d.	n.d.	n.d.	n.d.
10	Chrysene	n.d.	0.275	0.351	n.d.
11	Benzo (b) Fluoranthene	n.d.	n.d.	n.d.	n.d.
12	Benzo (k) Fluoranthene	n.d.	n.d.	n.d.	n.d.
13	Benzo (a) pyrene	n.d.	n.d.	n.d.	n.d.
14	Indeno (1,2,3-cd) pyrene	n.d.	n.d.	n.d.	n.d.
15	Dibenzo (a,h) anthracene	n.d.	n.d.	n.d.	n.d.
16	Benzo (g,h,i) perylene	n.d.	n.d.	n.d.	n.d.
	ΣPAHs	22.771	27.048	18.266	16.85

3. 15 Aliphatic Compounds in KCO WAF and CE-WAF

The profiles of aliphatic compounds between C_{12} and C_{34} are indicated in (Table 3-19) with their concentrations expressed as a percentage of total. The CE-WAF mixtures produced higher proportions of n-alkanes compared to KCO WAF. In the CE-WAF compared in the table, C_{25} carbon chains were consistently present than that which were found in KCO WAF indicating enhancement of aliphatic composition in WAF of dispersant treatment. In other words, CE-WAF's contained higher concentrations of carbons especially > C_{25} which was not witnessed in KCO WAF.

Table 3-19: Aliphatic compounds in WAF and CE-WAF solution

			Peaks as % of total			
Peak No.	**Carbon Chain**	**Compound Name**	**KCO WAF**	**Corexit® 9500 CE-WAF**	**Corexit® 9527 CE-WAF**	**Slickgone®NS CE-WAF**
1.	C12	Dodecane	6.614	6.642	5.903	3.565
2.	C13	Tridecane	12.768	6.470	10.354	9.158
3.	C14	Tetradecane	9.966	8.127	8.569	8.287
4.	C15	Pentadecane	9.073	7.786	8.067	7.491
5.	C16	Hexadecane	7.987	6.629	7.281	6.177
6.	C17	Heptadecane	10.258	8.860	9.653	8.373
7.	C18	Octadecane	7.122	6.308	6.789	6.324
8.	C19	Nonadecane	6.674	6.278	6.106	5.911
9.	C20	Eicosane	5.969	5.588	5.953	5.259
10.	C21	Heneicosane	6.722	6.235	5.936	5.147
11.	C22	Docosane	5.108	4.621	4.462	4.508
12.	C23	Tricosane	3.894	4.221	3.620	3.896
13.	C24	Tetracosane	3.916	3.682	3.556	3.716
14.	C25	Pentacosane	3.929	3.176	3.317	3.841
15.	C26	Hexacosane	-	2.875	2.504	3.627
16.	C27	Heptacosane	-	2.570	2.220	3.388
17.	C28	Octacosane	-	2.194	1.821	2.224
18.	C29	Nonacosane	-	2.541	1.477	2.378
19.	C30	Triacontane	-	1.491	1.307	1.921
20.	C32	Dotriacontane	-	1.027	1.103	1.394
21.	C34	Tetratriacontane	-	-	-	1.043

3. 16 Fourier Transform Infra Red Spectroscopy (FT-IR) Analysis

KCO WAF and CE-WAF samples analyzed by FT-IR showed a wide spectrum of results as indicated in (Table 3-20). FT-IR analysis indicated that TPH concentrations varied between the four KCO WAF and CE-WAF chemical mixtures. Corexit® 9500 CE-WAF had the highest TPH concentration (33.2 mg/L), followed by Corexit® 9527 CE-WAF (17.7 mg/L), then Slickgone® NS CEWAF (5.1 mg/L) and finally KCO WAF (2.14 mg/L) which was the chemical mixture with the lowest TPH concentration achieved.

Table 3-20: TPH concentrations by FT-IR for WAF and CE-WAF solutions

Chemical Mixture	TPH (mg/L)
KCO WAF	2.0
Corexit® 9500 CE-WAF	33.2
Corexit® 9527 CE-WAF	17.7
Slickgone® NS CE-WAF	5.1

3. 17 Stability of Serially Diluted KCO WAF and CE-WAF Solutions

KCO WAF and CE-WAF treatments were serially diluted in seawater and the fluorescence was directly determined in aqueous solutions and followed from 0 h to 96 h (Tables 3-21, 3-22 and Figure 3-5).

3. 17. 1 KCO WAF Fluorescence

At 0 h, fluorescence results were consistent since the fluorescence in each dilution (from 3.125% to 100%) was double that of the preceding one. At subsequent time intervals, the fluorescence value of various solutions increased and fluctuated then decreased at the 48 h which remained consistent at 72 h; and it decreased further at 96 h. In general, fluorescence for KCO WAF (100%) concentration decreased rapidly in the

first 24 h and then remained fluctuated around this value up to the 96 h. At 50% WAF dilution, no substantial decrease in the fluorescence was observed with time; and it remained in the same range up to 96 h. The other concentrations below that showed greater fluctuations in fluorescence at 24 h whereas at later periods stabilized fluorescence values were obtained (Table 3-21).

3. 17. 2 Corexit® 9500 CE-WAF Fluorescence

The changes of fluorescence in Corexit® 9500 CE-WAF in serial dilution for 96 h duration are indicated in Table 3-21, which have showed around 4 to 5-fold higher fluorescence compared to the fluorescence obtained from KCO WAF solution alone without the addition of oil dispersants. Moreover, serial dilutions of CE-WAF showed a decrease in fluorescence values with time accordingly. Maintenance of solutions showed greater loss in 100% CE-WAF compared to diluted WAF where the decrease in fluorescence value was of lower magnitude.

3. 17. 3 Corexit® 9527 CE-WAF Fluorescence

The increase in fluorescence of CE-WAF prepared with Corexit® 9527 as indicated in Table 3-21, was around 7-fold higher compared to KCO WAF; and it was around 2-fold more than of Corexit® 9500 CE-WAF. The pattern of decrease in fluorescence was the same for Corexit® 9527 CE-WAF to that found with Corexit® 9500. At 100% dilution the decrease in fluorescence was higher; and with dilution the percentage decrease was lowered, in other words, no decrease in fluorescence was obtained at lower dilutions.

3. 17. 4 Slickgone® NS CE-WAF Fluorescence

The fluorescence of CE-WAF prepared by treatment of KCO with Slickgone® NS oil dispersant was close to the value obtained with KCO WAF. Serial dilutions reduced the fluorescence accordingly. In addition, there was some decrease in fluorescence after 24 h and that fluctuated within the same range up to 96 h. At the lower dilutions fluorescence values was slightly higher than the 0 h value (Table 3-21).

As most of the serial dilutions values (3.125 to 50%) were negative as indicated in Table 3-22, the 100% values were selected for comparison between the four chemical mixtures as indicted in Figure 3-5. The four WAF and CE-WAF chemical mixtures had different percentage loss of test solution with Corexit® 9527 CE-WAF being the test chemical with the most percentage loss (60.0%), followed by Corexit® 9500 CE-WAF (40.0%), then Slickgone® NS CE-WAF (29.0%), then finally KCO WAF (26.0%) as the chemical with the lowest loss percentage over a 96 h test period (Table 3-22 and Figure 3-5).

Table 3-21: Change in fluorescence concentration using KCO WAF and CE-WAF's serial dilutions for 96 h duration

		Fluorescence					
		Dilution Concentrations (%)					
Chemical Name	**Time (h)**	**3.125%**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
KCO WAF	0 h	1.5	2.6	4.3	8.4	16.0	31.2
	24 h	0.9	32.6	24.0	47.7	17.2	22.2
	48 h	8.4	31.8	12.3	14.1	18.1	26.9
	72 h	8.2	29.1	11.5	13.9	17.8	26.4
	96 h	8.0	24.8	10.8	12.1	16.2	23.2
Corexit® 9500 CE-WAF	0 h	12.3	19.5	31.6	52.0	84.9	141.8
	24 h	10.1	15.1	24.0	39.7	65.9	106.2
	48 h	15.2	19.7	27.1	39.9	61.8	97.2
	72 h	14.5	18.7	25.5	37.7	58.0	92.1
	96 h	13.0	16.6	23.0	34.0	53.5	85.2
Corexit® 9527 CE-WAF	0 h	9.4	17.7	32.6	58.5	114.0	226.0
	24 h	8.6	13.8	25.4	45.9	88.4	166.5
	48 h	14.3	19.8	29.2	47.4	80.8	126.4
	72 h	13.6	18.9	27.1	43.5	73.1	103.5
	96 h	12.6	17.6	25.2	40.9	67.9	90.5
Slickgone® NS CE-WAF	0 h	3.1	5.2	8.9	16.6	31.5	57.8
	24 h	3.3	5.0	7.9	12.7	25.2	44.1
	48 h	11.1	12.5	14.8	19.1	29.7	46.1
	72 h	10.6	12.2	13.8	18.0	27.6	43.1
	96 h	10.4	11.7	13.8	17.6	26.8	41.1

Table 3-22: Concentration loss (%) for serial dilutions of KCO WAF and CE-WAFs

	WAF and CE-WAF Concentration Loss (%)					
Compound Name	**3.125%**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
KCO WAF	-44.2	-835.1	-150.6	-44.06	-1.01	25.6
Corexit® 9500 + KCO CE-WAF	-5.5	14.6	27.2	34.5	36.9	39.9
Corexit® 9527 + KCO CE-WAF	-33.9	0.71	22.7	30.0	40.2	59.9
Slickgone® NS + KCO CE-WAF	-241.7	-125.6	-55.7	-5.73	14.9	28.9

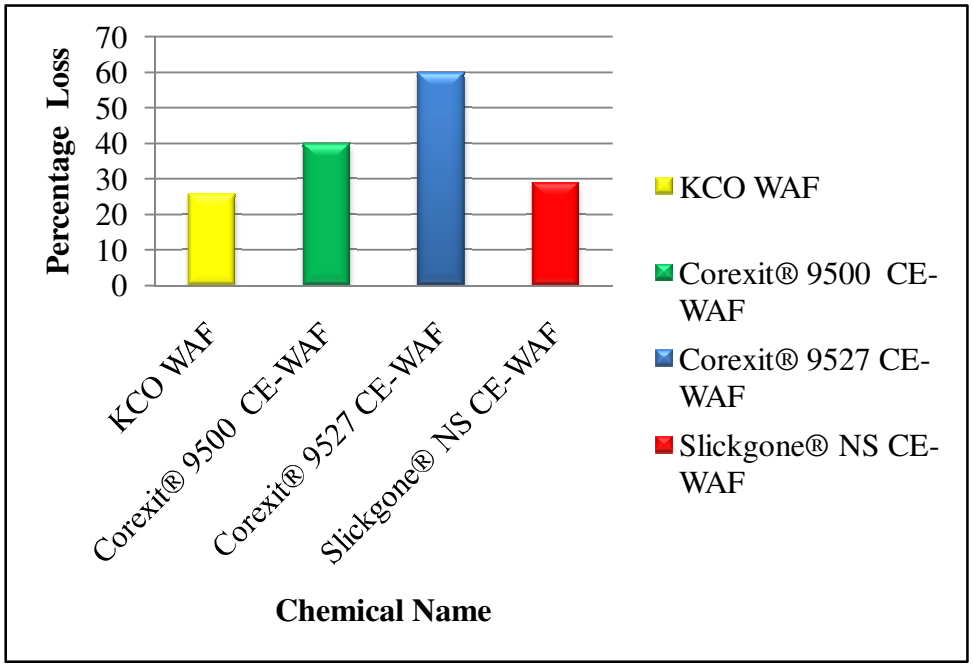


Figure 3-5: Percentage concentration loss for KCO WAF and CE-WAF solutions using 100% dilution concentration only

3. 18 Ultraviolet Spectrophotometry UV Analysis

The WAF and CE-WAF prepared in four replicates at a selected wavelength of 253 nm and their absorbance results are indicated in Table 3-23. The absorbance results of four chemical mixtures and seawater as indicated in Table 3-23 show clear differences in absorbance properties of each mixture. Corexit® 9527 CE-WAF produced the highest absorbance at 253 nm wavelength (0.355), followed by Corexit® 9500 CE-WAF (0.226), then by Slickgone® NS CE-WAF (0.0915), and finally KCO WAF (0.0652) as the mixture with the lowest absorbance.

Table 3-23: Absorbance of KCO WAF and CE-WAF at 253 nm wavelength

No.	Chemical Name	Absorbance (at 253 nm Wavelength)	Mean/SD/SE
1	KCO WAF	0.0652	0.065/0.023/0.012
2	Corexit® 9500 CEWAF	0.226	0.226/0.072/0.036
3	Corexit® 9527 CEWAF	0.355	0.355/0.149/0.075
4	Slickgone® NS CEWAF	0.0915	0.092/0.028/0.014

SD: Standard deviation, SE: Standard error.

3. 19 Discussion

3. 19. 1 Experiment No. 1

KCO WAF prepared from variable oil loadings resulted in different BTEX concentrations in WAF solutions. When oil loadings increased, the BTEX concentrations also increased but were not proportional to the oil loadings. The 10.0 g KCO/L seawater loading resulted in a generally less BTEX values compared to that of the lower loadings. This decrease in VOCs with increasing oil to water ratios was explained as a" depletion effect", in other words, oil becomes depleted in water soluble material ultimately leading to a decrease in the apparent solubility. Others have also encountered difficulty in WAF preparation, which reveal that; oil to dissolving medium ratios did not increase the TPH content in WAF (Navas et al., 2006; Gonzales-Doncel et al., 2008). Among all BTEX compounds, toluene was found to be the highest in WAF prepared in this study. In an earlier study (Shui et al., 1990), three different crude oils (Western Sweet Mixed Blend, Prudhoe Bay crude and Southern Louisiana) were used at similar oil-to-water ratio (1:1000). The concentration of benzene, ethyl benzene and dimethylbenzene became less considerable in the total WSF analyzed; while toluene became the VOC's component with the highest concentration similar to the findings in this study as indicated in Plate 3-1, A. Similar to BTEX, TPH concentrations were also increased with the increasing oil to water loadings, but were not proportional to the oil loadings (plate 3-1, B). Singer et al., (1998) have concluded that total hydrocarbon concentrations (THC_{C7-C30}) generally have increased with increasing oil loadings (1.01-25.7 g/L).

3. 19. 2 Experiment No. 2

The effect of two mixing speeds (300 and 650 rpm) and two mixing times (24 and 120 h) on BTEX and TPH concentrations resulted in a variable values attributable to different mixing energies and mixing durations. The data showed that at low mixing speed, by increasing the mixing time to 120 h, the BTEX concentration in WAF is doubled than that found at 24 h but the pattern of each of the BTEX compounds remained the same. The data at higher mixing energy and 24 h mixing duration resulted in slightly higher BTEX concentrations compared to that found in WAF prepared at the low mixing energy for the same duration. Increasing mixing duration at high speed brought no appreciable change in BTEX concentration (Palte 3-1, A). TPH concentration was approximately the same in WAF prepared at higher speed at the two mixing durations. Therefore, a significant difference in BTEX concentrations was noticed between the two mixing duration using a low mixing energy as there was an increase in BTEX concentrations when mixing duration increased (Plate 3-1, B). Generally, low mixing speed produced close TPH concentrations than that of higher mixing speed for the two mixing durations. Thus, a low mixing speed for two mixing durations (300 rpm with 24 and 120 h) is more representative in terms of BTEX and TPH concentrations. Blenkinsopp et al., (1996) recommends that a low-mixing speed method is preferred were no vortex is generated and the resultant WAF is more replicable.

3. 19. 3 Experiment No. 3

Mixing time is considered as an essential parameter during the preparation of WAF of crude oil in order to achieve a substantial level of "functional equilibrium" within the preparation flask. In other words, the aqueous phase resulting from mixing the crude oil with seawater should be saturated in terms of present soluble petroleum hydrocarbons compounds (Singer et al., 2000). In Exp. No.2, the difference between two mixing times for WAF preparation indicated that the BTEX values for the 24 h mixing durations seemed to be half the values obtained at the 120 h mixing time. This suggested that longer mixing durations at low mixing speed can allow more of the volatile fraction becomes partitioned in WAF resulting in higher concentrations. TPH concentrations didn't seem to be much affected by the change in mixing time as they

were less sensitive. Therefore, BTEX results in Exp. No.3 were consistent with the findings in Exp. No. 2.

The several mixing times using 1 g/L of KCO WAF preparation resulted in different BTEX and TPH concentrations in which BTEX concentrations fluctuated during the various selected mixing times. The different mixing durations resulted in different BTEX concentrations in which, toluene > benzene > xylene > ethylbenzene. However, the pattern was the same with all BTEX compounds, which demonstrated a gradual increase starting from the first 30 min up until the 24 h of mixing time then a decline was observed in which benzene demonstrated the most appropriate pattern consistent with increasing mixing durations until 24 h as indicated in Plate 3-2, A. TPH concentrations demonstrated a linear relationship and a consistent pattern with the selected mixing times, in which it increased from the first 30 min until the 24 and 48 h of mixing. TPH concentration attained saturation within the 24 h of mixing duration. Unlike BTEX results, TPH values produced a more reliable understanding of how the selected oil loading of 1 g KCO/L seawater behaved when exposed to various mixing durations until reaching saturation point as indicated in Plate 3-2, B. Thus concluding that 24 h time interval is the most appropriate mixing time to generate a reproducible WAF solution. Earlier studies by Singer et al., (2000), demonstrated that data generated from flame ionization gas chromatography (GC/FID) showed no substantial difference between 24 h and longer mixing times such as 72 h; and concluded that functional saturation could be achieved with Prudhoe Bay crude oil at most oil loadings with 24 h mixing period. The same author discouraged using mixing periods beyond 24 h because of the possibility of the commencement of bacterial action. And since the aqueous solubility of crude oil in seawater is normally around 30 mg/L, the concentration of crude oil dissolved in a water-soluble fraction prepared after a gentle stirring for 24 h was in the range of 0.5-24.04 μg/mL which is in agreement with TPH results obtained in this study (Boylan and Tripp, 1971; Anderson et al., 1974; Blackman and Law, 1980; Pearson et al., 1981; Sophia and Balasubramanian, 1992; Ali et al., 1995; Saeed et al., 1997).

3. 19. 4 Experiment No. 4

KCO WAF and CE-WAF were prepared under identical conditions and compared for their compositions. The four prepared chemical solutions produced different BTEX results which reflected the different abilities of each chemical to solubilize each of the BTEX compounds in the WAF solution. Slickgone® CE-WAF had the highest benzene and toluene concentrations, but their concentrations were the same for Corexit® 9500 and Corexit® 9527 CE-WAFs. Ethylbenzene and xylene concentrations were higher in CE-WAF of Corexit® 9527 > Slickgone® CE-WAF > KCO WAF > Corexit® 9500 CE-WAF as shown in Plate 3-3, A.

TPH concentrations varied for the four chemical mixtures analyzed in which Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > KCO WAF which demonstrated the effect of chemical dispersant on dispersing more oil droplets in the aqueous phase and therefore increasing the TPH concentration (Plate 3-3, B).

BTEX concentrations were variable for all of the four chemical mixtures, although benzene and toluene produced a similar pattern; the same thing was obtained for ethylbenzene and xylene, which behaved similarly in terms of their concentrations in the four chemical mixtures. Other studies on Kuwait crude oil using different oil-to-water ratio and different preparation method have obtained volatile fraction concentration equivalent to 0.0353 mg/L, which was much higher that what was achieved in this study 0.00214 mg/L as total BTEX fraction. BTEX concentrations obtained in this study where approximately to what Saeed et al., (1998) have reported earlier. Differences in BTEX values might be attributable to methodology differences in which Lockhart et al., (1984) have used higher mixing speed, less mixing time (2 h) and more settling duration (48 h). Other studies have also indicated that the aromatic fraction of 10% hydrocarbon water-accommodated fraction (WAF) extracted from Kuwait crude oil was dominated by benzene and toluene which was similar to our findings (NRC, 1985). These findings will ultimately lead us to conclude that the variable BTEX concentrations in the four chemical treatments can be attributable to multi-component solubility of each BTEX compound in aqueous solution and the volatility of individual BTEX compounds during preparation.

TPH concentrations behaved in a manner, which seems logical in a sense that all of the three CE-WAF preparations had a TPH concentration higher than that of KCO WAF. But, within the three CE-WAF preparations each one produced different TPH

concentration. Studies conducted by Singer et al., (1998), have demonstrated that total hydrocarbon content (THC $_{(C7-C30)}$) (TPH plus BTEX) for Corexit®9527 CE-WAF solutions was higher relative to THC obtained from WAF solutions of Prudhoe Bay crude oil; at any given oil-loading ratio. More specifically, the volatile fraction (BTEX) in the case of WAF solutions; were found to be composed of an average of 95.9% volatiles, whereas Corexit®9527 CE-WAF yielded only 67.0% volatiles.

The other type of investigation to determine the effect of delivery of test chemical in the vortex (DIV) or delivery not in the vortex (DNIV) indicated that BTEX concentrations fluctuated in a similar range for the two preparation conditions and no consistent pattern was deduced from it. As for TPH concentrations, the (DNIV) conditions resulted in higher concentrations than the ones obtained for the first preparation condition (DIV). TPH concentrations were the highest for Corexit®9527 CE-WAF > Corexit®9500 CE-WAF > Slickgone® CE-WAF > KCO WAF. Hence, the second preparation condition (DNIV) was adopted because it yielded higher TPH concentrations. Singer et al., (2000) found that the two methods resulted in a dispersion which behaved similarly, but the resulting chemical mixtures had different concentrations. When they used similar oil loading to the one used in this study, similar concentration behavior for the two methods was noticed; with the prevortexed method resulting in higher concentrations.

The effect of stirrer bar size on BTEX and TPH concentrations indicated that for 2.5 cm bar size BTEX concentrations were double than what was obtained after using 3.5 cm stirrer and the increase in magnetic bar size didn't increase BTEX concentrations. However, TPH concentration obtained after using 2.5 cm stirrer was less than that of 3.5 cm bar size and TPH difference between the two sizes was minimal and not significant. As BTEX concentrations had irregular patterns in most of the characterization experiments, TPH provided a better understanding of chemical preparations behavior and the two magnetic bar sizes produced a stable and uninterrupted vortex with the selected mixing speed (300 rpm) with minimal differences in TPH concentration. Tsvetnenko et al. (2002) examined the influence of magnetic stirrer dimensions on several mixing speeds in different chemical preparations and concluded that increasing magnetic bar length increased the depth of the vortex at each mixing speed, but test results were not verified by BTEX and TPH analysis.

From the generated data it can be deduced that the chemical mixtures (WAF and CE-WAFs) exert various influences on the ability of BTEX compounds to be solubilized in aqueous solution as each of the three oil dispersants have different

degrees of dispersing crude oil and accommodating it in the water phase during laboratory preparations and field applications. Due to the volatility of BTEX compounds during preparation and sampling, each of the four chemical mixtures resulted in different concentrations. Similarly, due to the structural complexity of KCO WAF and the three CE-WAF preparations, TPH concentrations were different again.

3. 20 Individual PAHs in KCO WAF and CE-WAFs

Individual polycyclic aromatic hydrocarbons were determined in WAF and CE-WAF of the four chemical mixtures prepared. For KCO WAF, seven out of sixteen PAH compounds were determined, and individual PAH compounds were detected in which they constitute part higher molecular weight PAHs, but most of them were not determined. Application of Corexit® 9500 and Corexit® 9527 dispersants in CE-WAF treatments further enhanced individual PAHs levels and changed the composition of PAHs in both treatments. For the WAF and CE-WAF preparations which had the highest total PAHs was as follows: Corexit® 9500 CE-WAF > KCO WAF > Corexit® 9527 CE-WAF > Slickgone® CE-WAF. Individual PAH concentrations were variable among the four test chemicals, for instance; naphthalene concentration was the highest in KCO WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > Corexit® 9527 CE-WAF. Only chrysene was detected in Corexit®9500 and Corexit® 9527 CE-WAFs, but they were not detected in other test chemicals. Phenanthrene was present at higher concentrations only in Corexit® 9500 and Corexit® 9527 CE-WAFs treatments but was found low in other test chemicals. In general, Corexit treatments increased PAHs concentrations and only PAHs concentration in KCO WAF was close to that of (Corexit® 9527 CE-WAF) and the lowest was in PAH concentration was Slickgone® CE-WAF. When a comparison was conducted by Saeed and Al-Mutairi (2000), they demonstrated that Kuwait crude oil export quality contained relatively low amounts of PAHs in the WSF solution.

Since the composition of the WSF of Kuwait crude oil was the subject of many studies, what should be carefully considered here as that crude oil having the same name don't necessarily have identical chemical composition as the one investigated in this study. And the composition of the mixed oil for export purposes depends mainly on consumer specification, refining process, types of different crude oils; which might lead to either high or low PAH values (Anderson et al., 1974; Ali et al., 1994; Saeed et al.,

1998; 2000). Couillard et al., (2005) have also concluded that the addition of dispersants (Corexit® 9500 CE-WAF) have caused an increase in the concentration of total PAH when weathered Mesa Light crude oil was used. And when a WAF solution was prepared form oil loadings similar to the one used in this study, total individual PAH were 11-folds much higher than what was obtained in this study. The most probable reason can be related to the type of crude oil (light crude oil) used in their study in which the addition of oil dispersants have increased the dissolution of some of the PAHs rather than others compared to the Kuwait crude oil used in this study which is of a mixed blend from different oil fields. In addition, in the case of CE-WAF solution which was prepared from different oil loadings (0.05-0.5 g/L), total individual PAH were still higher than our findings. Moreover, Couillard et al. (2005), findings indicated that dispersants noticeably increased the aqueous concentrations of higher molecular weight PAH (with three or more benzene rings) which were less water-soluble. Several studies have indicated that the addition of dispersants to oil slicks have influenced the introduction of PAHs into the water column by increasing the concentration of higher molecular weight PAH making them more bioavailable to fish because they are more persistent in the marine environment (Anderson et al., 1974; Rice et al., 2001; Yamada et al., 2003). Other studies have demonstrated that chemical analysis of KCO have indicated that almost a quarter of the total chemicals present in this crude oil were aromatic hydrocarbons (21.9%) in which within this percentage, naphthalene comprises 0.7% of the aromatic fraction (NRC, 1985).

When Cohen et al., (2001) used a ratio of 1:30 (dispersant-to-oil) but with different test oil with much higher oil loadings than the one used in this study; he found a five-fold increase in total PAH concentrations in Corexit® 9527 CE-WAF when compared with WAF of crude oil alone. In this study, total PAH concentration in Corexit® 9527 CE-WAF was less than what was obtained for KCO WAF bearing in mind much lower oil loading was used in our case and the reason can be attributed to the amount of water soluble components partitioned in the aqueous phase. Moreover, Singer et al., (1998) have concluded when Prudhoe Bay crude oil was used, higher total hydrocarbon content (THC C7-C30) values were detected in Corexit® 9527 CE-WAF solution relative to WAF concentrations, at any given oil loading ratio. Barron and Ka'aihue (2003) have indicated that dispersant efficacy may vary as a function of the composition of the dispersed oil; of the energy used to mix the chemical solution and the type of dispersant used; and this reflect the variation among the test chemicals analyzed in this study.

3. 21 Aliphatic Compounds in KCO WAF and CE-WAFs

The profiles of aliphatic compounds between C_{12} and C_{34} and their chromatograms are indicated in Plate 3-4 and the chromatogram peaks represents the retention time for aliphatic compounds detected in KCO WAF or CE-WAFs samples. The CE-WAF mixtures produced higher proportions of *n*-alkanes especially above C_{25} compounds that were not detected in KCO WAF. Saeed et al., (1998) have indicated that, straight chain aliphatic hydrocarbons (*n*-alkanes) were present in KCO WSF solution in decreasing quantities. Generally, the highest percentage of aliphatic compounds was detected in the following: Corexit® 9500 CE-WAF > Slickgone® CE-WAF > Corexit® 9527 CE-WAF > KCO WAF; in which these compounds were barely detected in KCO WAF as indicated in Plate 3-4.

3. 22 TPH Analysis

Results were distinct when TPH analysis was conducted by two methods, Fourier Transform-Infra Red (FT-IR) and spectrofluorophotometry (SPM) for the four test chemicals. TPH by FT-IR method revealed that, the highest TPH concentration recorded was for Corexit® 9500 CE-WAF > Corexit®9527 CE-WAF > Slickgone® CE-WAF > KCO WAF. But TPH by spectrofluorophotometeric method, indicated the highest TPH concentration recorded was for Corexit®9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > KCO WAF (Plate 3-5).

3. 23 Ultraviolet Spectrophotometry UV Analysis - Absorbance

The concentration patterns achieved from ultraviolet spectrophotometry analysis (UV) produced different absorbance results when various KCO WAF and CE-WAF's were analyzed. In a sense that the highest absorbance recorded among all chemical mixtures was as follows: Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > KCO WAF. It can be clearly understood from the differences in UV absorbance, that oil dispersants can increase the absorbance of the WAF with respect to the different absorbance obtained among the three CE-WAF mixtures. KCO WAF without the addition of oil dispersants had the lowest absorbance, which indicates that oil dispersants increase the probability of oil molecules to enter the water phase and thus increasing its absorbance, which can lead to an increase in toxicity levels in the marine ecosystem (Plate 3-5).

3. 24 Fluorescence Analysis

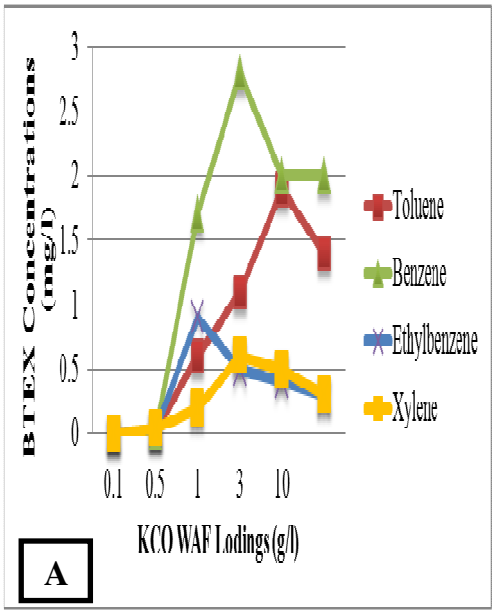
Fluorescence concentration patterns were consistent with the ones obtained from absorbance in a sense that highest fluorescence concentration obtained was as follows: Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF >KCO WAF (Plate 3-5).

Generally, FT-IR, UV (absorbance), fluorescence and TPH concentrations results were consistent with each other as a pattern was deduced in a sense that all CE-WAF solution resulted in higher concentrations than KCO WAF solution did. Although,

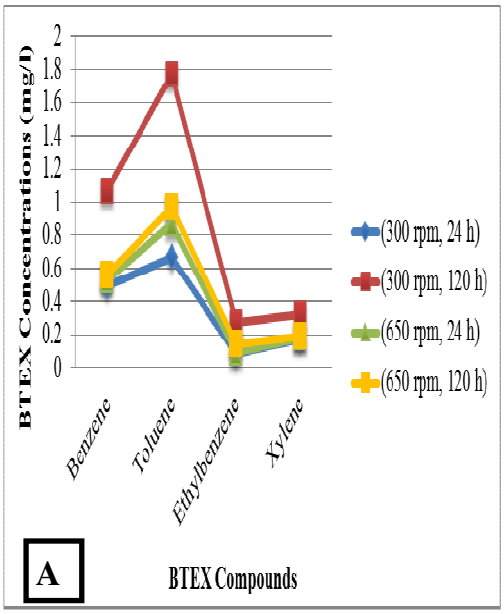
there is some discrepancy between the results obtained from the FT-IR method on one side and the results obtained from spectrofluorophotometer, UV and fluorescence on another side (Plate 3-5).

3. 25 Stability of Serially Diluted WAF and CE-WAF Solutions

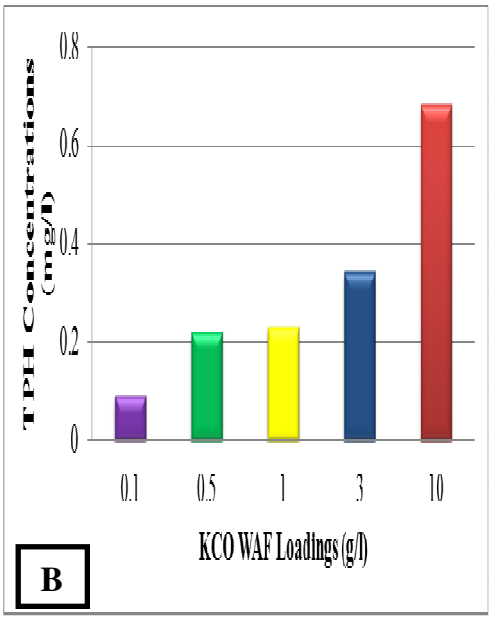
KCO WAF and CE-WAF chemical mixtures had different percentage loss of test solution, in which the test chemicals with the most percentage loss over a 96-h test period were as follows: Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > KCO WAF. Each of the four chemical mixtures was susceptible to test solution loss, which clearly indicates the sensitivity of each test solution in abiotic conditions to the environmental surroundings of the test experiments. Each of one of the chemical mixtures produced various loss of solution through evaporation during 96 h exposure duration in serial dilution settings. As the 100% dilution was considered here for comparison between the four chemical preparations, apparently the action of oil dispersants produced more absorbance (fluorescence) than what was observed in KCO WAF. The test chemical which had the highest fluorescence in 100% dilution was as follows: Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > KCO WAF. KCO WAF seemed to have a consistent pattern and no observable loss was noticed compared to that of the CE-WAFs which had a gradual decrease from 0 to 96 h. This can be reflected in natural environment where there is even a greater loss of overall consistency of chemical solution due to the various environmental factors such as temperature, wave action, dissolution and etc.



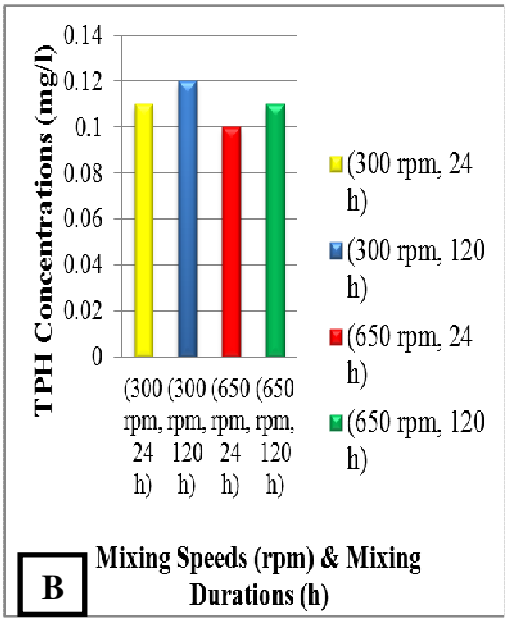
Experiment No. 1



Experiment No. 2

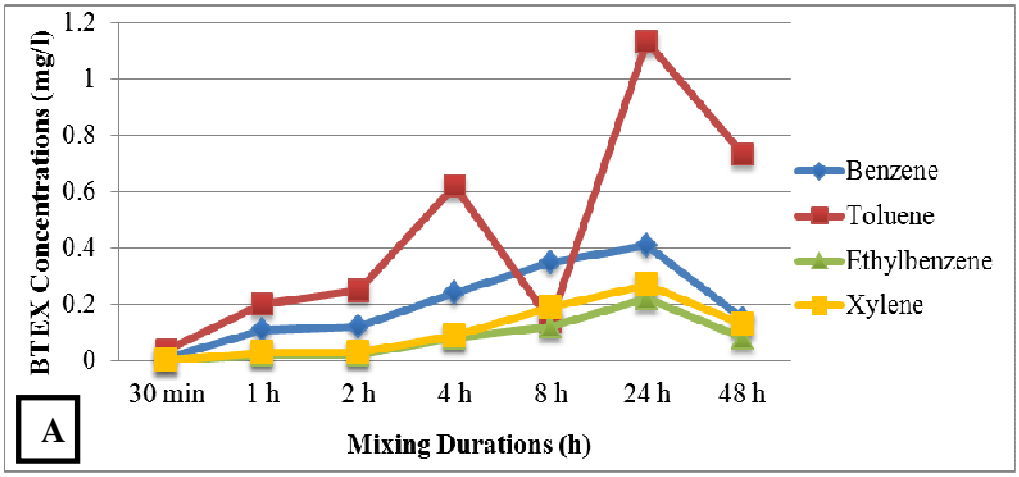


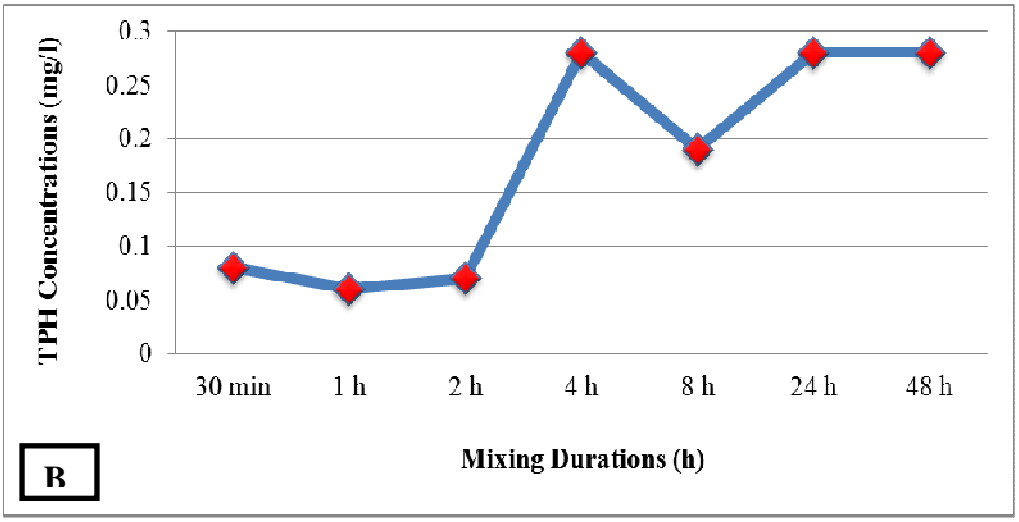
Experiment No. 1



Experiment No. 2

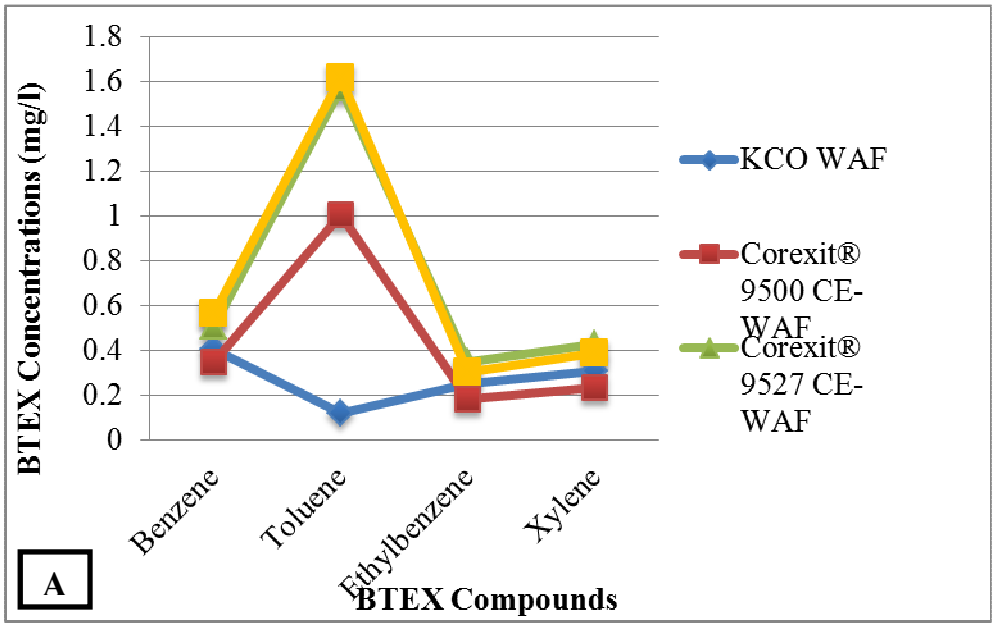
Plate 3-1: Experiment No. 1 (A) BTEX concentrations at various KCO WAF loadings, (B) TPH concentrations at various KCO WAF loadings; Experiment No. 2: (A) BETX concentrations for KCO WAF preparation at various mixing speeds and mixing times, (B) TPH concentrations for KCO WAF preparation at various mixing speeds and mixing times.

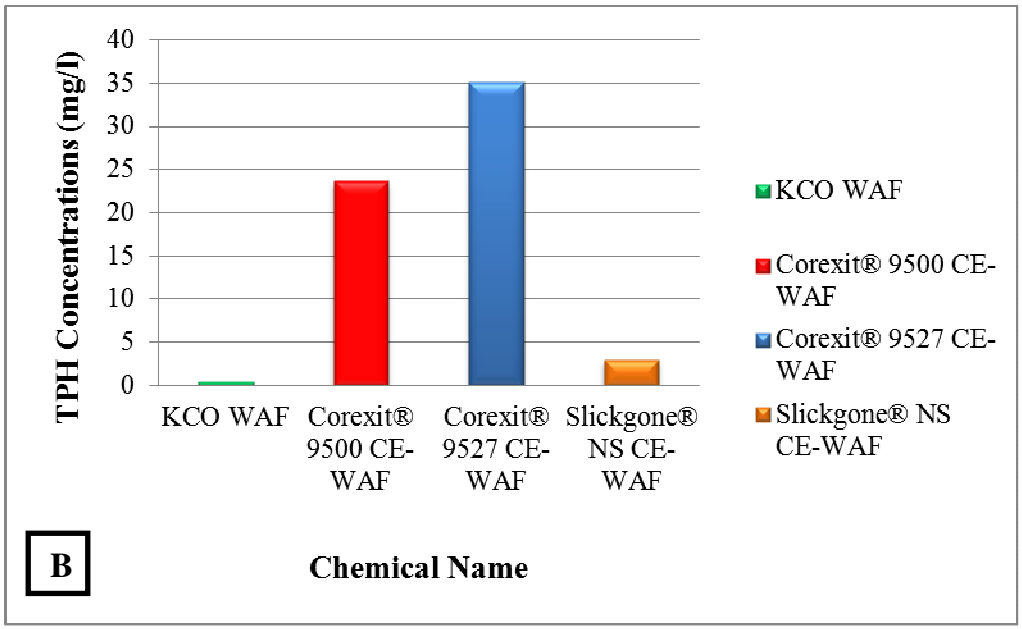




Experiment No. 3

Plate 3-2: Experiment No. 3 (A) Effect of several mixing times on BTEX concentrations for KCO WAF preparation; (B) Effect of various mixing times on TPH concentrations for KCO WAF preparation.





Experiment No. 4

Plate 3-3: Experiment No. 4 (A) Average BTEX concentrations for various KCO WAF and CE-WAF solutions; (B) Average TPH concentrations for KCO WAF and CEWAF solutions.

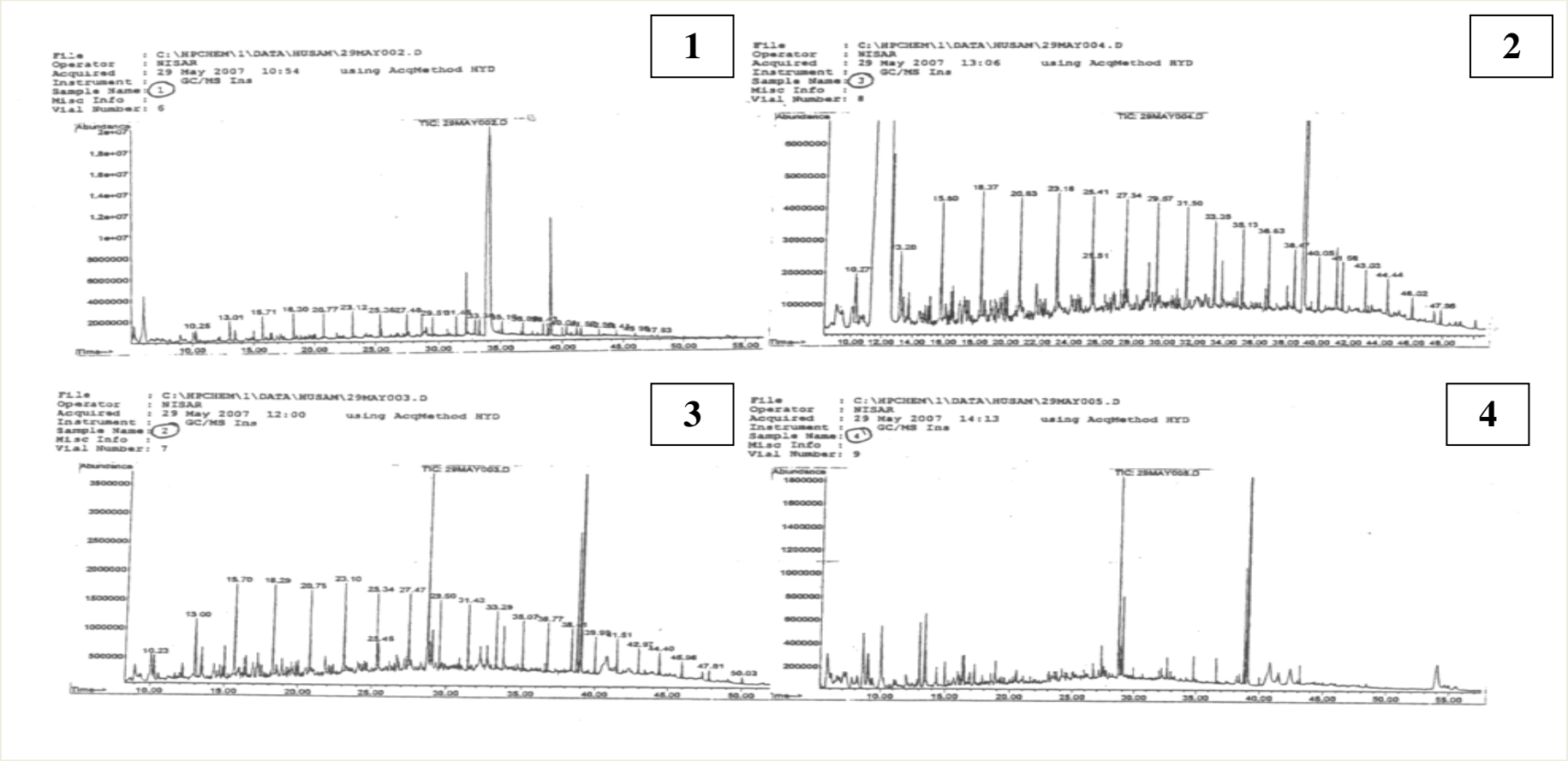
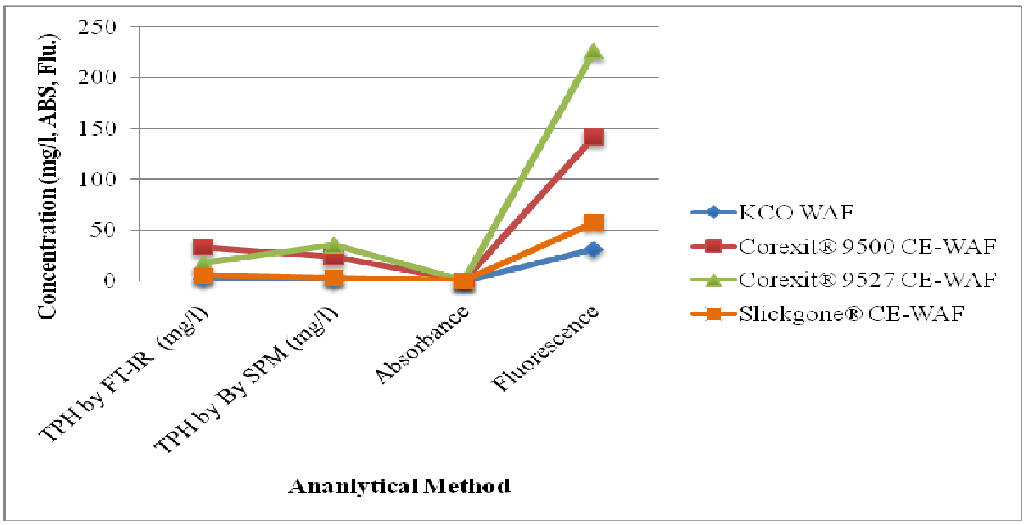


Plate 3- 4: Chromatograms of aliphatic compounds for: 1) KCO WAF, 2) Corexit® 9500 CE-WAF, 3) Corexit® 9527 CE-WAF and 4) Slickgone® CE-WAF treatments.



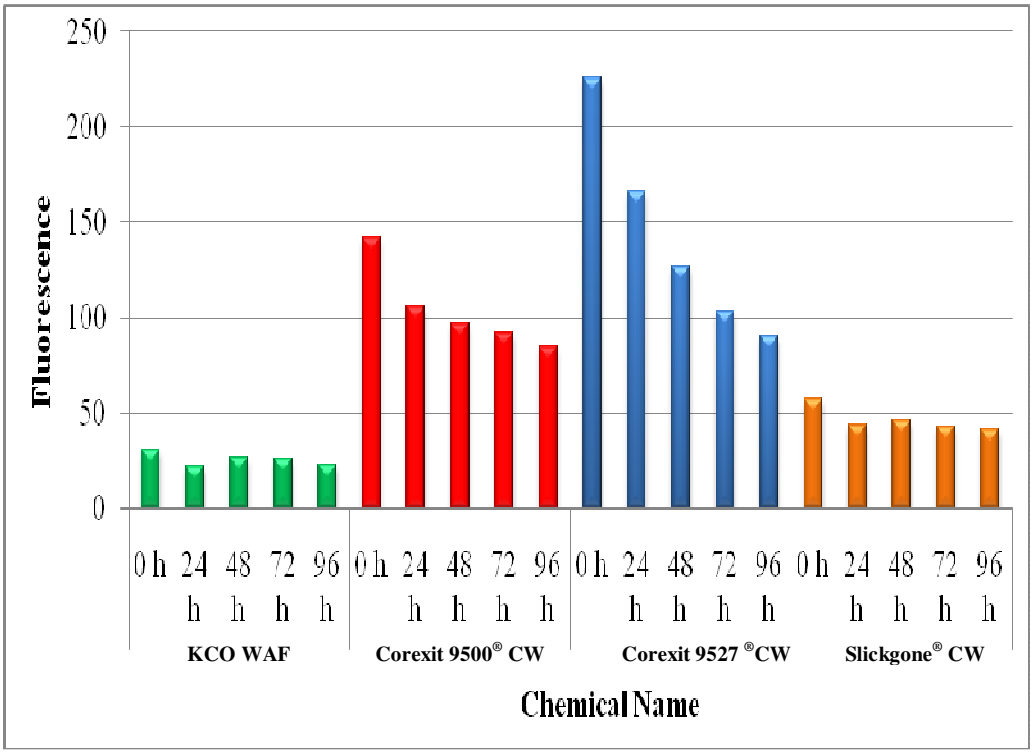


Plate 3-5: (A) Comparison between four analytical methods: TPH by FT-IR, TPH by SPM, Absorbance, and Fluorescence for KCO WAF and CE-WAF quantification; (B) Percentage concentration loss for KCO WAF and CE- WAFs 100% dilution concentration only.

3. 26 Conclusion

Dissolution of crude oil in an aqueous medium such as seawater can be influenced by many factors, which might ultimately affect the whole stability of the resultant WAF/ or CE-WAF solution. Maintenance of the stability of a laboratory prepared WAF solution is rather difficult and the outcome solution differs greatly than the one formed during natural processes such as in the case of an oil spill incident. Many investigators have attempted to duplicate natural environmental conditions in controlled laboratory settings for achieving natural mixing mechanisms of oil to induce solubility in seawater. In this study, several chemical analyses were conducted to characterize and understand the behavior of selected test chemicals in our laboratorial conditions. Variable oil loadings were examined during the preparation of WAF solution, and its composition varied greatly which made dose selection for toxicity testing rather difficult. Therefore, a single oil loading was selected and subsequent serial dilutions were made. The variable oil loadings experiments yielded different BTEX results; the decrease in VOCs with increasing oil to water ratios was because of depletion effect (evaporation) as oil becomes depleted in water soluble material ultimately leading to the apparent decrease in oil solubility. BTEX and TPH results increased with increasing oil loadings but were not proportional with oil loadings, and some BTEX compounds had higher concentrations than others in WAF loadings thus producing irregular pattern. As for the effect of two mixing speeds and two mixing times on BTEX and TPH concentrations, it resulted in a variable values attributable to different experimental settings. The results suggested that longer mixing durations at low mixing speed can allow more of the VOC (BTEX) to become partitioned in WAF resulting in their higher final concentrations. TPH concentrations were not affected much by the changes in experimental conditions. In general, when several mixing durations were selected using 1 g KCO loading, it resulted in fluctuating BTEX concentrations which demonstrated a gradual increase starting from the first 30 minutes up until the 24 h of mixing duration then a decline at 48 h. TPH concentrations resulted in a linear relationship, which attained saturation at the 24 h and it was concluded that this duration is the most appropriate to achieve a more reproducible and stable WAF solution. And when the effect of two stirrer bar sizes on BTEX and TPH concentrations were examined it revealed that BTEX concentration were decreased by increasing stirrer bar size, but TPH concentrations had a minimal and not significant increase while increasing magnetic stirrer size.

Moreover, when KCO WAF and CE-WAF of KCO plus three oil dispersant formulations were prepared to examine the effects of oil dispersant on the dispersal of KCO, BTEX concentrations resulted in more variable patterns for all of the four chemical mixtures and didn't completely describe the difference between WAF and CE-WAF of KCO, thus the dispersion of KCO with different dispersants led to obtain different TPH and BTEX concentrations. TPH concentrations were higher in CE-WAF solutions compared to that obtained for WAF alone. So, from the generated data it can be deduced that different dispersants have different capacities of dispersing oil in water column.

Other results obtained from chemical analyses conducted using total petroleum hydrocarbons (TPH) by FT-IR and spectrofluorophotmetery methods, UV (absorbance), fluorescence, polycyclic aromatic hydrocarbons (PAHs) and aliphatics; indicated that they were consistent with each other in which CE-WAF solution resulted in higher concentrations than in WAF solution. Although there is some discrepancy between the results obtained from the pattern achieved, it confirmed that CE-WAF concentrations were higher than the ones obtained for WAF solution.

As the variable oil loadings were tested, the results were not proportionally increased with increased oil loading. Therefore, in this research, 1 g KCO/L seawater was the selected oil loading which was implemented because higher loadings had no additional advantages. And since the aqueous solubility of crude oil in seawater is normally around 30 mg/l, the concentration of crude oil dissolved in a water-soluble fraction prepared after a gentle stirring for 24 h was in agreement with other values reported in literature. In comparison to our study, the values obtained after preparing a WAF solution at a ratio of 1 g KCO/L seawater, TPH was 0.5 mg/L which was enhanced after the addition of oil dispersant (CE-WAF) at 10% of the oil volume. Although the comparative value of all of the previous results is generally questionable even in literature because of the complex differences in preparation, methodologies, extraction techniques, between laboratories and perhaps most importantly, the specific conditions associated with the release of hydrocarbons into the water mass, this lead to the formulation of a general conclusion that CE-WAF treatments increased the TPH, individual PAH compounds, and aliphatic hydrocarbons. The understanding of the behaviors of WAF and CE-WAF solutions will enable us further to predict its role when it is used in toxicity testing against native marine organisms and the usage of oil dispersants in combating oil spills.

CHAPTER FOUR

FISH TOXICITY TESTING

4. 1 Introduction

The toxicity of crude oil is considered collectively as the toxicity of the organic and inorganic chemical mixtures present within, and which constitutes crude oil. Because crude oil is composed of thousands of compounds, its toxicity is further interpreted as the fraction of crude oil which can induce most effects on marine fish species. The aromatic fraction of petroleum is considered as the most toxic fraction to marine organisms, since various forms of polycyclic aromatic hydrocarbons (PAHs) and their breakdown products such: as 9,10-anthracenedione, benz [a] anthracene-7, 12-dione, and 1(3H)-isobenzofuranone can be carcinogenic, mutagenic or toxic (Lehto et al., 2003; Billiard et al., 2008). More specifically, the majority of PAH in crude oil are mono- aromatic hydrocarbons composing of two- and three- ring aromatics which can be considered as toxic and can induce adverse effects. The high- molecular weight PAHs which are composed of four to seven rings are significantly mutagenic, teratogenic and/ but less toxic to fish species than low-molecular weight hydrocarbons (Scannel et al., 2005). Others have contributed the toxicity of petroleum to its most soluble and volatile fraction such as: benzene, toluene, ethyl benzene and xylene (BTEX) compounds which are highly mobile in the environment and soluble in seawater, thereby increasing the possibility of exposure to marine fish species. Moreover, benzene is classified as an A class carcinogen by the U.S. EPA (NOVA Chemicals®, 2008). Other studies have demonstrated that low-molecular weight hydrocarbons which are relatively water-soluble and not persistent in the marine environment because they can be easily evaporated, they can penetrate cell membrane, become toxic and cause narcosis. Meanwhile, high-molecular weight hydrocarbons which are considered to be less water soluble, but more persistent; are more toxic than the low-molecular hydrocarbons (Di Toro et al., 2000; Rice et al., 2001). The sole purpose of this compound classification is to define the subset of chemicals from crude oil and seawater mixtures (WAF) as the "worst-case" in terms of its toxicity to marine fish species (Irwin, 1997). The water soluble fraction (WSF) of crude oil contains a mixture of PAHs, phenols, and heterocyclic compounds containing nitrogen or sulfur (Saeed and Mutairi, 1999) and more toxic volatile compounds that can be quickly

absorbed in fish from the WSF with adverse consequences to biological organization (Collier et al., 1996).

Although oil, dispersants are utilized as an effective means to combat oil spills, specifically in marine waters, there still exits numerous concerns about the toxic effect of dispersed oil on marine organisms such as fish, which live in the water column (Otitoloju, 2005; Venosa and Holder, 2007; and Nyman et al., 2007). In addition, decision makers are still concerned about the toxicological and environmental consequences associated with crude oil and the application of dispersants to combat oil spills. Therefore, the combined impact of crude oil plus oil dispersants on aquatic organisms must be assessed by regulatory agencies as an integral part of the pre-approval process for these dispersant formulation to be applied as a response strategy to oil spill (Pace and Clark, 1993). Therefore, three dispersant formulations, which were provisionally registered to be used in the Arabian Gulf, were obtained from their manufacturers for investigating its relative combined toxicity with crude oil. Water-accommodated fractions (WAFs) of Kuwait crude oil alone, and chemically-enhanced water accommodated fractions (CE-WAF) of Kuwait crude oil plus three individual oil dispersants were prepared by variable oil loading using a series of decreasing concentrations of Kuwait crude oil and the three individual oil dispersant in sea water according to CROSERF protocols (NRC, 2005). In addition, the effects of WAF produced by variable loading and by single loading followed by serial dilutions were examined to determine which of the two methods result in a more toxic test medium. Other factors involved in the preparation of WAF test solution like headspace, mixing energies, mixing duration and percent vortex were kept constant by obtaining the same type of magnetic stirrer plates, mixing vessel, and the same size of Teflon® coated stirrer bar (NRC, 2005).

In order to compare the relative toxicities of the selected oil and oil plus individual oil dispersant preparations, acute bioassays were selected as an appropriate test system. This assists in further understanding the behavior of such chemical mixtures and the induced toxic effect on marine fish early-life stages in a dose-response exposure system. The conducted acute bioassay tests use mortality as an end point of the test and as a measure of toxicity which are expressed as LC_{50} (mg/L), the lethal concentration affecting 50 % of the population tested, in other words causing mortality over pre-determined exposure time (i.e., 24, 48, 72 & 96 h). In addition, it serves as a numerical indicator of the toxicity of a test chemical such as KCO WAF and CE-WAFs (Polisini and Miller, 1988). The rationale for specifying a test period which encompasses a 96 h

(acute test) is that, this test provides a reproducible, cost efficient and rapid concentration curves for determining the toxic effects of test chemicals such as crude oil on marine fish. And through the calculation of LC_{50} (mg/L) concentration which measures one specific biological response that is death, which is the first step in the risk assessment of hazardous chemicals; other measurements can be included which provides a better understanding of the case such as: solubility and partitioning of test chemical in the aqueous phase, degradation rate and which fraction of the test chemical causes the majority of the toxicity. Also, it by toxicologist as the most highly rated test system, for evaluating the adverse effect of a hazardous chemical in water mediums such as: warm, cold, acidic, basic and hard) and to organisms which represents different trophic levels of the marine ecosystem (Johnson and Finley, 1980). Wilson (1977) indicated that starvation of fish larvae could increase their vulnerability to oil toxicity. Vosyliene et al., (2005) observed increased rainbow trout (*Oncorhynchus mykiss*) larval mortality rate at the end of the test after exposure to water-soluble fraction of crude oil. That is why reporting of 24 to72-h LC_{50} is utilized in this study since mortality achieved at the end of the bioassays (96 h) could possibly be induced by starvation rather than by the toxic action of test chemicals as by 96 h; morphological development of fish larvae should have taken place and more likely there were attempts by fish larvae to search for food.

4. 2 Exposure Methods

There are two toxicity tests to examine the combined effects of oil and oil dispersants on marine organisms. Each exposure method has a specific objective in which one method ensures that the relative toxicity of oil/dispersant mixture is no greater than the individual toxicity of crude oil alone in a sense that the presence of two chemical additives (oil and dispersant) in the same fluid (seawater) can lead to synergistic effects (Spinelli and Lucas, 2006). And since crude oil is composed of a mixture of thousands of compounds, the toxicity of individual compounds is known but the toxicity of crude oil, refined products and oil dispersants can be extremely difficult to measure because researchers have limited knowledge about the synergistic and additive effects of chemical mixtures (Overton et al., 1994; Baek et al., 2004). The second aims to guarantee that the toxicity of dispersant alone is not greater than the toxicity of oil alone (DEFRA, 2006). As the relative toxicity classification system of

toxic substances as indicated by (USFWS, 1984; Hunn and Schnick, 1990) for aquatic fish using a 96-h LC_{50} and it states that: 100-1000 mg/L (Practically Nontoxic), 10-100 mg/L (Slightly Toxic), 1-10 mg/L (Moderately Toxic), 0.1-1.0 mg/L (Highly Toxic, and <0.1 mg/L (Extremely Toxic).

Over the years, several toxicity exposure methods have been developed in order to assess the toxic effects of unknown chemical substances that might pose certain hazards to aquatic fish species. Toxicity exposure regimes for oil and dispersed testing should in a sense reflect environmental realism, in other words persistence of spilled oil in marine environment. Of those methods:1) static, 2) semi-static, and 3) continuous flow-through exposure systems have been implemented to further understand the behaviour of test chemicals in WAF solutions and the response of marine fish to such mixtures. A static exposure system is used when exposure concentrations are expected to remain within 80-100% of the nominal over the 96-h exposure period. The other test regime (Semi-Static) is used when exposure concentrations are expected to remain within 80-100% of the nominal values by renewing the test solutions every 24-h period for the whole exposure duration. Continuous-flow through exposure system can be used effectively in toxicity testing when test concentrations are expected to decline from nominal values by approximately more than 20% over a 24-h exposure period (OCED, 2000).

4. 3 Fish Resources of Kuwait

Fisheries are considered as an integral part of traditional heritage in Kuwait, and it represents the second most significant natural resources next to oil and the most vital natural resources in the Arabian Gulf region (Carpenter et al., 1997). It has a significant economic value since the fishery industry offers major investment opportunities for the private sector. In addition the fishery resources sustain a major recreational fishing (FAO, 2003). Generally, the Arabian Gulf has low diversity of species and there are about 130 fish species are known to occur in Kuwait Territorial Waters (Krupp and Muller, 1994). The main reason for the economic success of fishery's industry in Kuwait is the strong market demand. Nevertheless, the market demand of seafood especially fish in Kuwait continue to far exceed the sustainable potential of local fish resources, leading to an expansion in the aquaculture industry in Kuwait and the supply

of fresh fish that the traditional capture fisheries are unable to provide. The aquaculture production included fresh water species such as tilapia in fish farms and marine aquaculture systems which are composed of 74 floating marine sea cages in the western part of Kuwait bay. That made Kuwait and Iran the only two countries in the Arabian Gulf region with commercial mariculture activities operated since 1992 with production started since 1994 with 50 t sold (Al-Hossaini, 1999). The annual landings (t) for capture fisheries from 1990 through 1997 for Kuwait ranged from 4,042 to 7,826 t and the decrease in catch rates for some of fish species can be attributed to over-fishing. The mean abundance of fish larvae collected from Kuwaiti waters with 300 µm mesh nets during 2000-2001 was 36-281/m^3, and the peak spawning periods of fish (eggs and larvae) produced a good correlation pattern with peak zooplankton abundances (Micheal et al., 1986). The annual fish catch for yellow-fin sea bream (shea'am) 282 t, the annual landings of mullet from 1996 to 2001 averaged 686 t.

4. 4 Test Species

Selection of test species for aquatic toxicity testing should follow certain criteria according to Perkins (ADEC, 2000), which identifies it as a candidate species for toxicological evaluation and assessment of hazardous chemicals, such as: 1) species seasonal availability, 2) social and economic values, 3) sensitivity to crude oil, 4) ecological relevance, and 5) practicability of test. Fish was selected as a test species in this study because of the following: 1) species were cultured in the aquaculture facilities at Kuwait Institute for Scientific Research and available for conducting readily toxicological studies, 2) fish embryonated eggs and larvae serve as different developmental stages exposed to marine pollution in a sense that fish eggs are floating like the zooplanktons and don't have the ability to escapes from the oil spill. On the other hand once eggs hatch, fish larvae they will be able to swim away from the oil slick tend to be more sensitive because they no longer have he protection of the egg enveloping membrane against eternal stressors, 3) fish have an economic importance to the country and understanding its sensitivity to marine pollutants will further assist in its protection, 4) there is a difference in sensitivity to crude oil between the fish and invertebrate species as pelagic fish are the most sensitive animals to oil pollution and intertidal invertebrates are the most resistant. Because intertidal organisms have adapted

their bodies to withstand the rigorous and intertidal hydrocarbon stresses by burrowing, closing their shells, reducing their metabolism rate (Rice et al., 1979; Moles, 1998).

In the literature, many fish species have been selected for toxicological assessment of oil and dispersed oil, for instance salmon, flounder and larval Pacific herring have been evaluated for their sensitivity to Prudhoe Bay and Alaska North Slope crude oil under non-CROSREF test conditions (Rice et al., 1976; Anderson, 1985; Moles, 1998; Barron et al., 2003). More specifically, the selection of certain species should be more closely related to the selection of specific life stage such as an embryo or larval stage. For instance, herring larvae are recommended in literature over herring embryos because of similar sensitivity, more toxicity responses and more rapid bioaccumulation of toxins in species tissues and organs, making them more ideal for assessing and comparing the bioavailability of oil and dispersed oil in marine environment according to Perkins (ADEC, 2000).

In this study, three fish life stages were selected for acute toxicity tests which are: embryonated egg stage, larvae hatched during exposure to test chemicals and pre-hatched larvae. The rationale for selecting three developmental stages was that we wanted to investigate which stage is more sensitive to KCO WAF/ CE-WAFs. Fish embryos are considered to be very useful fish life stage especially in toxicological studies since they possess uniform spherical shapes which make it simple and easy to handle. They also, don't ingest material from surrounding environment because they are not yet developed, have minimum surface area in contact with ambient seawater, and they don't have external organs like setae and gills (Carls et al., 2008).

4. 5 Objectives

The objectives of these studies were to: 1) determine the toxicity of WAF of Kuwait crude oil (mix blend-export quality), CE-WAF of Kuwait crude oil + Corexist® 9500 dispersant, CE-WAF of Kuwait crude oil + Corexist® 9527, and CE-WAF of Kuwait crude oil + Slickgone® dispersant, 2) contrast the comparative relative toxicities of the selected species to Kuwait crude oil and dispersed oil, and 3) compare the joint toxicity of crude oil plus oil dispersants for each of the selected species.

4. 6 Materials and Methods

Fisheries are considered as an integral part of traditional heritage in Kuwait, and it has a significant economic value since the fishery industry offers major investment opportunities for the private sector. In addition, the fishery resources sustain a major recreational fishing. The main reason for the economic success of fishery's industry in Kuwait is the strong market demand. Nevertheless, the market demand of seafood especially fish in Kuwait continue to far exceed the sustainable potential of local fish resources (FAO, 2004).

Local fish species sobaity-sea bream (*Sparidentex hasta*), hamoor-orange-spotted grouper (*Epinephelus coicoides*), maid-mullet (*Liza klunzingeri*) and juvenile shaem-yellow-fin sea bream (*Acanthopagrus latus*) were selected to conduct the 96-h toxicity bioassay (Plate 4-1). The rationale for selecting four different species in this study is that they constitute an important food source for local consumption and because of their significance to the State of Kuwait and the Arabian Gulf region as an economically important fish species. Also, they are considered to be of the most expensive species and highly priced sea food and the local market demand exceeds their supply; that is why they were selected for culture (Hussain et al., 1981). Embryonated eggs, larvae hatched during exposure and pre-hatched larvae were obtained from the hatchery of Mariculture and Fisheries Department (MFD) at Kuwait Institute for Scientific Research. In general, water quality parameters for the fish species used in this study in the MFD hatcheries were: dissolved oxyegen (5-6 mg/L), temperatyre (20-28°C), salinity (40-42 ppt), and pH (8.2-8.6).

4. 6. 1 Sobaity - Sea Bream

Sobaity-sea bream (*Sparidentex hasta*) (Valenciennes, 1830) is considered one of the most significant commercial fish in Kuwait and the Arabian Gulf region, it possess dark grayish color with silver color on top and pale on the ventral side and it has an external fertilization. Its average length ranges from 30.9-75 cm and can be found at depths of 10 to 50 meters. It has a wide distribution, which can range from only the Arabian Gulf to the southern tip of India and in coastal waters and in between the reefs, but is very rare in Kuwaiti reefs. Its main diet is crustaceans and some other kinds of

fishes and it has a spawning period, which ranges from February to March (Bauchot and Smith, 1984; Carpenter et al., 1997; Lone et al., 2003 (Plate 4-1, A).

4. 6. 2 Hamoor – Orange -Spotted Grouper

Hamoor-orange-spotted grouper (*Ephinephelus coicoides*) (Hamilton, 1822) is another important commercial fish in the Arabian Gulf region, its color is tan on the upper body and whitish on the ventral side with many brownish orange spots distributed on its head and body. This species is very common in the Arabian Gulf region, which can exist in a variety of habitats ranging from sandy to rocky bottoms and it has external fertilization and its average length ranges from 18-95 cm. Moreover, it can be seen in coralline areas from shallow areas to the greater depths in the Arabian Gulf. It's common in some Kuwaiti islands such as Kubbar and Umm Al-Maradem which have a coralline habitat and can survive on sandy flats adjacent to reef areas which it can excavate and rest in small depressions. It has a wide distribution around the world, which can range from the tropical, subtropical and warm-temperate Indian Ocean regions. In addition, it can be found in the West Pacific to the Ryukyu Islands in Fiji to the northern New South Wales. Hamoor primarily feeds on crabs, fish, shrimps and cuttlefish. Its spawning period is from March to June and its biology has been studied extensively and, more specifically its aquaculture potential is being successfully developed at the Mariculture and Fisheries Department of the Kuwait Institute for Scientific Research (Plate 4-1, B). (Heemstra and Randall, 1993; Carpenter et al., 1997).

4. 6. 3 Meid-Mullet

Meid-Mullet (*Liza Klunzingeri*) (Day, 1888) fish species belong to the mugilidae family and have prominent greenish grey color on the dorsal side which shades to silver color on the ventral side. Its average length ranges from 11.6-20 cm. It is distributed mainly in the Arabian Gulf to India and in the Western Indian Ocean. Meid has an elongated body shape and with external fertilization it has two spawning periods, which range from January to March and from October to December (Breder and Rosen, 1966; Randall, 1995; www.fishbase.org, 1995) (Plate 4-1, C).

4. 6. 4 Shea'am -Yellow-Fin Sea Bream

Shea'am-Yellow-Fin sea bream (*Acanthopagrus latus*) (Houttuyn, 1782) belongs to the Sparidae family. It has external fertilization of eggs and it feeds mainly on mollusks, echinoderms, crustaceans and worms. Yellow-fin sea bream occurs in shallow waters and it enters estuaries and river mouths so it can exist in demersal, marine, brackish and freshwater environments. Yellow-fin sea bream become mature from year one and two, and its average length ranges from 27.3-45 cm. It has a wide distribution in the world which ranges from the Indo-West Pacific: Arabian Gulf and along the coast of India to the Philippines, south to Australia and north to Japan and its spawning period is from February to March (Bauchot and Smith, 1984; Okiyama, 1988; Buxton and Garratt, 1990; Lone et al., 2003(Plate 4-1, D).

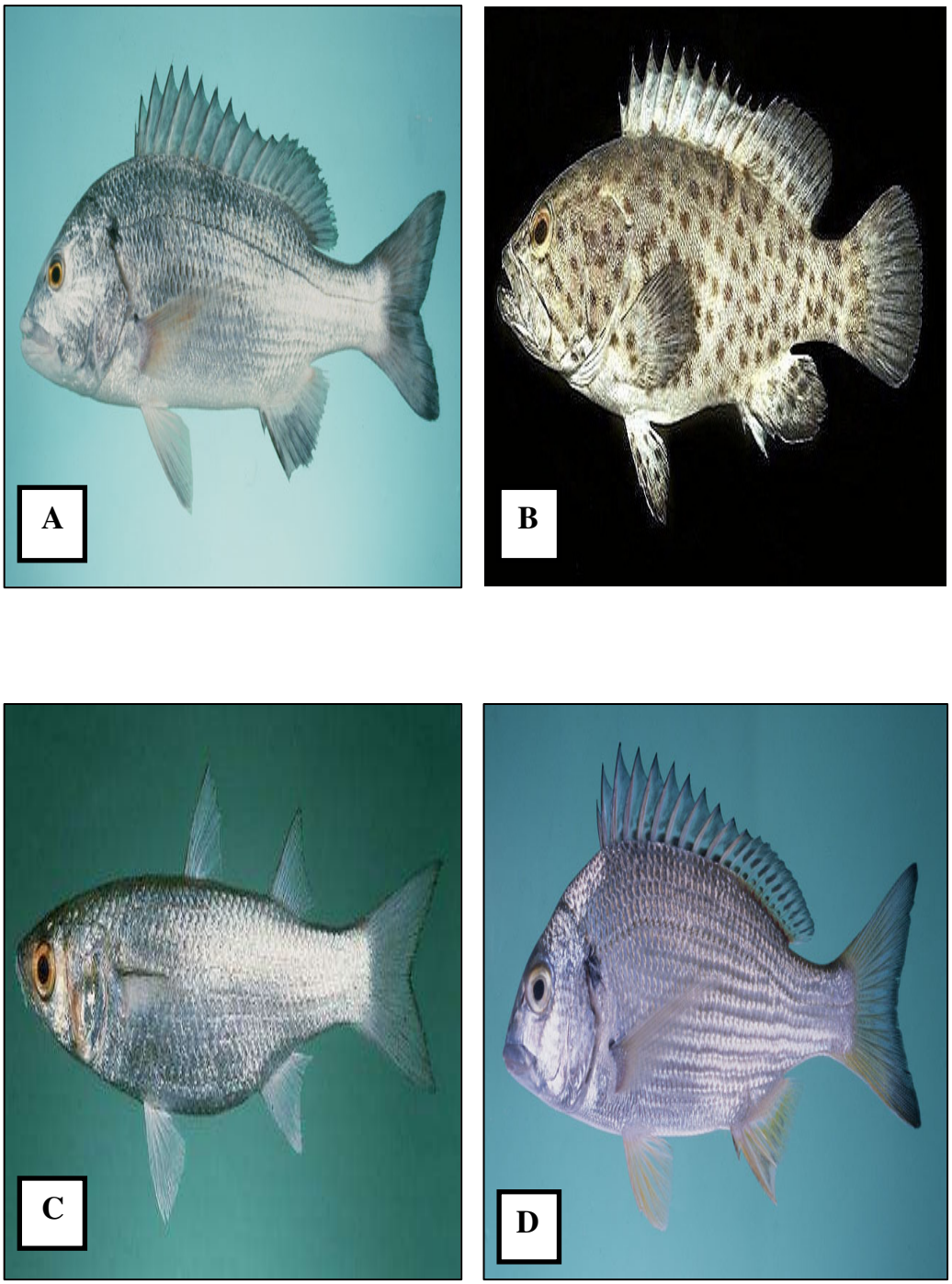


Plate 4-1: (A) Sobaity-sea bream, average length ranges from 30.9-75 cm (from Randall, 1997), (B) Hamoor-orange-spotted grouper, average length ranges from 18-95 cm (from Randall 1997), (C) Meid-mullet, average length ranges from 11.6-20 cm (from Randal, 1995), and (D) Sheam-yellow-fin sea bream, average length ranges from 27.3-45 cm (from Randall, 1997).

4. 7 Acute Toxicity Tests

A 96-h acute toxicity tests were conducted following OCED Guideline for the Testing of Chemicals - Fish Embryo Toxicity (FET) Test (OCED, 2006). The method using solutions prepared from water-accommodated fractions (WAFs) of: 1) Kuwait crude oil alone (KCO WAF), 2) Kuwait crude oil + Corexist® 9500 dispersant (Corexist® 9500 CE-WAF), 3) Kuwait crude oil + Corexist® 9527 (Corexist® 9527 CE-WAF), and 4) Kuwait crude oil + Slickgone® dispersant (Slickgone® CE-WAF). Solutions were prepared the by two methods: 1) variable oil loading using a series of decreasing concentration of Kuwait crude oil, and 2) by single loading and subsequent serial dilutions of KCO alone, and KCO + three individual oil dispersant in sea water according CROSERF protocols (NRC, 2005). Kuwait crude oil was procured from Petroleum Research Division at Kuwait Institute for Scientific Research, (export quality – mixed blend) type and stored in amber bottles in dark and room temperature (specifications included in Chapter Two).

Dilution water (seawater) which was used to make serial dilutions of WAF solution was obtained from the same holding tank that fish larvae and embryonayed eggs reared in. Seawater was filtered with 0.45-µm Whatman® filter paper, aerated with pure oxygen for 15 minutes until saturation prior to test. Water quality parameters of fish holding tanks and test dilution water were measured using WTW® 350i water quality probe (Table 4-1). Dilution water was aerated for 15 minutes prior to conducting the bioassay.

Preparation of test solutions depends on the purpose of the test such as estimation of 96-h LC_{50}. At least five test concentrations plus a control solution should be prepared with an appropriate geometric dilution series selected in which each successive concentration is about 50% of the previous one such as: 100%, 50%, 25%, 12.5% and 6.25%. The selected concentrations will assist in the accurate calculation of LC_{50} concentration and its 95% confidence intervals (Report EPS, 1990). Five serial dilutions of Kuwait crude oil WAF and Kuwait crude oil plus three individual oil dispersants CE-WAF was made in a 100 ml glass beakers and made up to a final volume of 50 ml of exposure medium (WAF/ CE-WAF). Serial dilutions were made in a multiple replicates with non-toxic controls (only filtered seawater) for each replicate (Figure 4-1). The five concentrations and a control exposure design are the minimum number of concentrations required to meet the statistical requirements of the test (OCED, 2006).

Static toxicity (non-renewal) test was conducted for 96 h with the following fish developmental stages: (a) embryonated fish eggs (E) brought from the hatchery after 24 h of their release, (b) larvae hatched during the toxicity test (LHE) from the same embryonated eggs exposed in the same test, (C) pre-hatched larvae (PHL) which was exposed to test chemical after 24 h of hatching in the hatchery. Fish larvae were not fed throughout the exposure period. The main reason for not feeding the test organisms is that yolk sac nourishes fish larvae for three days and the oil globule further nourishes the same larvae for an additional two days. In the case of embryonated eggs, they were washed and checked for complete fertilization and toxicity tests were initiated at times from 4 to 8 h post-fertilization. As for bioassay involving pre-hatched larval stages, initiation of toxicity was conducted 24 h after hatching. The weight of an egg and larvae is normally about 0.75 and 0.10 mg, respectively. A minimum of 10 to 30 fish (embryonated eggs or pre-hatched larvae depending on availability) were placed using a glass pasture pipette (wide mouth side) in 100 ml glass beakers.

A preliminary range-finding test was performed to define the specific oil and dispersed oil concentrations (dose selection) to be used in a definitive toxicity test, and other test conditions like ambient room temperature (26°C) and photoperiod were kept constant and no aeration was introduced in the conducted toxicity tests. Test solutions including dilution control water were not renewed during the 96 h duration of the test (static non-renewal test). Dead fish larvae were daily removed (24-h interval) once the count of survived fish was taken and fish which were not responsive to gentle prodding with pasture pipette were considered dead (Fuller et al., 2001). The end point of toxicity test was fish mortality; therefore tests were terminated after 96 h. Glass beakers were covered with Parafilm® to eliminate contamination from external sources and any accidental spillage of exposure medium.

Table 4-1: Water quality parameters of dilution water

Parameter	Value
Dissolved Oxygen	1.7-3.0 (mg/L)
Temperature	28.0-29.0ºC
pH	7.0-8.0
Salinity	38.0-39.8 ppt
Conductivity	59-60 mS/m

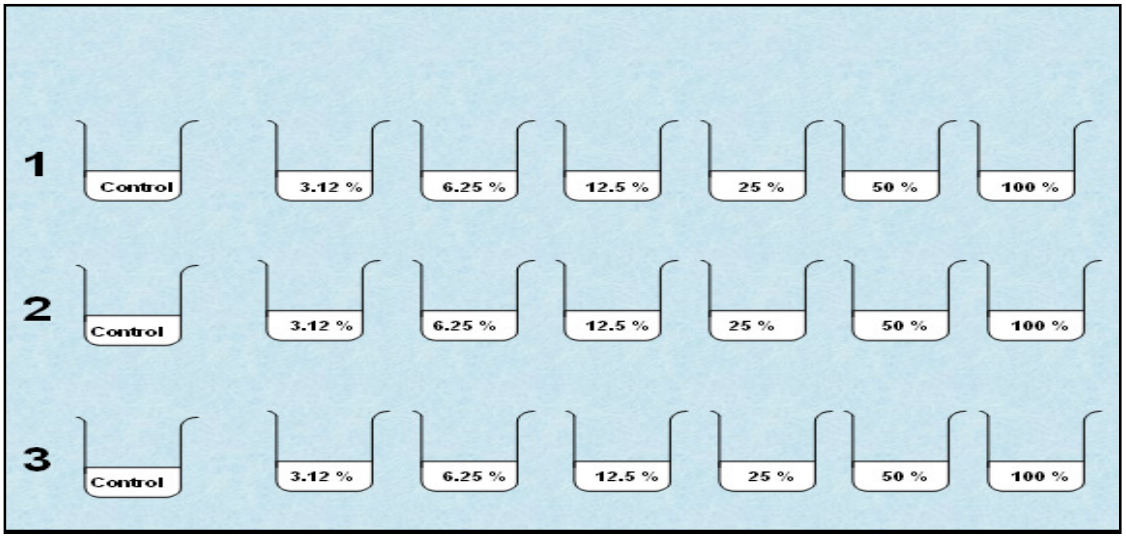


Figure 4-2: Serial dilutions of WAF/ CE-WAF solutions and controls

4. 8 Morphological Abnormalities in Sea Bream Fish Larvae After Exposure to Crude Oil

Developmental abnormalities in sea bream larvae were examined daily during exposure to (1 g KCO WAF/L seawater) in a standard static 96-h acute toxicity test under the stereomicroscope with a high magnification and were photographed for documentation. KCO loading of 1 g KCO/L seawater was selected because it was demonstrated previously that this loading was the most toxic to fish larvae and further examination of other sublethal effects of KCO WAF such as developmental abnormalities in fish larvae can assist in understanding the toxic behavior of KCO. Fish larval survival and types of deformities were recorded simultaneously during exposure to KCO WAF. Deformity types have been classified as follows: Type-A- Lordosis (V-Shaped, inward curvature of the spine), Type-B-Kyphosis (hunch back), Type-C-Scoliosis (lateral bending of the spine), Type-D-Irregular body shape, and Type-E-Deformed caudal fin. Alterations of growth or development were mapped for spinal deformation, yolk sac deformity or edema, fin fold, head and body size according to Jezierka et al. (2000). Controls (filtered seawater) were run with each set of exposure experiments for quality control in assays without any toxicant present in the medium of exposure, and to compare it with larvae exposed to toxic mixtures.

4. 9 LC_{50} Determination

The LC_{50} value for each day of exposure was determined by Lethal Concentration Estimation Program Version 1.0, Copyright© 1990-1995, Institute for Inland Water Management and Waste Water Treatment RIZA (Lelystad, The Netherlands) and programmed by Modelco (Endinhoven, The Netherlands) by order of RIZA. The software for LC_{50} determination estimates the statistical parameters and before the estimation starts, the spreadsheet is validated for the following properties. The survivor spreadsheet is a rectangle. If numbers outside this rectangle were entered, no estimation is performed. The size of the concentration and time vectors must have the same dimensions as the number of columns or the number of the rows of the survivor rectangle respectively. The time vector consists of at least two items; the first item has to be zero. The concentration vector consists of at least two Items; the first item must be a blank. This means that the concentration is equal or smaller than 1E-6. The time vector and the concentration vector have ascending elements. The columns of the survivor rectangle have ascending elements also. In time the number of survived organisms cannot increase.

The parameters of the statistical model are estimated. The statistical model used is the log-logistic function.

S(C,t;A,mu,b,lc50(t))=A*{(lc50(t)/C)^(1/b)}/{1+(lc50(t)/C)^(1/b)}*exp(-mu*t),

S: The expectation of the number of survived organisms.

C: The concentration of the toxic.

A: The initial number of organisms.

mu: The natural mortality parameter.

b: log-logistic parameter, which determines (indirectly) the slope of the function.

lc50(t): log-logistic parameter, which determines the concentration at which only 50% of the organisms are alive.

t: time.

The initial estimation is performed with Spearman-Karber parameter estimation (Hamilton et al., 1977). Then, the parameters are iterated with The Maximum Likelihood method and the Newton-Raphson method according to Kooijman (1980). Toxcalc version 5.0 statistical packages (Copyright© 1994-1996 Tide pool, Scientific

Software and Micheal A. Aves. All rights reserved, USA) was used to calculate the no observable effect concentration (NOEC).

4. 10 Statistical Analysis

All statistical analyses were conducted using Minitab® Statistical Software - Version 15© 2006 by Minitab Inc. All rights reserved. General Linear Model (GLM) was used to determine whether or not exposure concentration (%) and exposure time (h) exerted a significant effect on fish egg hatching and/or larval survival during toxicity test. The GLM include three procedures: regression, the analysis of variance (ANOVA), and the analysis of covariance (ANCOVA), and it function on the premise that it predicts one variable (dependent); in our case is the hatching or larval survival success response from one or more variables (independent) like exposure concentration (%) and exposure time (h). Finally, p values were calculated and effects were considered significant if ($p<0.05$) and effects were considered not significant if ($p>0.05$). Mean, standard deviation (SD) and standard error (SE) were calculated for biological experiments using Windows Excel®. Standard deviation was calculated to show how much variation there is from the mean and standard error was calculated to show the extent in which the sample mean differs (±) from the population mean.

4. 11 Research Constraints

Certain experimentation constrains were encountered during the course of this research study with test organisms. Species tested have different breeding seasons; therefore obtaining the same fish age (egg and/or larvae) for all four test species was rather difficult to achieve for all the bioassays conducted. The most available fish species for this study were sea bream and orange-spotted grouper, but juvenile yellow-fins sea bream and mullet were less frequently available and were used for a few experiments for the purpose of screening their sensitivity and response to KCO WAF-CE-WAFs toxicity. Most of the experimental bioassays conducted with sea bream pre-hatched larvae and orange-spotted grouper embryonatged eggs, larvae hatched during exposure and pre-hatched larvae. For

the bioassays, eggs of 24 h old age were used and tests conducted with larval stages were initiated within 10 h of hatching time. Juvenile yellow-fins sea bream and mullet fish were used for other bioassays during the course of this study.

4. 12 Results

4. 12. 1 Sobaity- (Sea Bream)

4. 12. 1. A Egg Hatching Success in Control Seawater

Comparison of sea bream egg hatching and larval survival success was conducted in order to determine how sea bream eggs and larvae behaved in five control seawater treatments under laboratory conditions. Sea bream embryonated eggs normally take 24 to 48 h for successful hatching in control-filtered seawater. Except in a few cases, most of the eggs hatched in the first 24 h, and by 48 h the remaining eggs had hatched in most of the controls. In the preliminary studies, embryonated eggs hatched successfully, but the survival of hatched larvae was variable and ranged from 33-61% by 96 h and larvae may have died from starvation. A better representation of larval survival percentages can be noticed at 72-h of exposure (Table 4-2).

Table 4-2: Sea bream egg hatching and larval survival success in control seawater

	Control Replicates (C:Controls)					
Exposure Time (h)	**C1**	**C 2**	**C 3**	**C 4**	**C 5**	**Mean/SD/SE**
0-h Total Eggs	30	30	30	30	28	
24-h Total Hatched Eggs	29	30	29	30	28	29.2 /0.84/0.37
24-h Egg Hatching Success %	97%	100%	97%	100%	100%	
48-h Total Surviving Larvae	28	29	28	29	27	28.2/0.84/0.37
48-h Survival Success %	93%	97%	93%	97%	96%	
72-h Total Surviving Larvae	16	20	24	23	26	21.8/3.90/1.74
72-h Larval Survival Success %	53%	67%	80%	77%	93%	
96-h Total Surviving Larvae	11	16	10	13	17	13.4/3.05/1.36
96-h Larval Survival Success %	37%	53%	33%	43%	61%	

SD: standard deviation, SE: standard error.

It was revealed that extreme care is required in transferring the eggs to exposure vessels and these precautions improved the survival percentages of freshly hatched larvae up to 96 h in revised experiments (Table 4-3). In a revised study, 12 replicates of controls were run to ensure the conditions for successful hatching of eggs

and the survival of hatched larvae in experimental conditions. The main changes were, care in transferring the eggs to test vessel from the stock, using the same quality of water that was used for the breeding, and pre-oxygenation of water for 15 min prior exposure to control seawater. The physicochemical properties of water are described in other section and the best conditions were followed in all the later experiments.

The data in (Table 4-3) shows that in twelve controls, egg hatching percentages ranged from 59-100% at 24 h, with 8 replicates having hatching success close to 80% and above. At 48 h, egg hatching further improved with only one treatment having 75% hatching and the rest were above 80%. Larval survival declined by 15% at the 72 and 96 h, and finally at 96 h larval survival percentage ranged from 69-100%.

The variation in egg hatching and larval survival during control exposure reflects the quality of eggs at the initiation of the experiment, since not necessarily all the embryonated eggs were of good and suitable quality to render the bioassay valid. The data suggests that with each experiment seawater blank is essentially required; however other conditions of the assay were suitable to run the test.

Table 4-3: Sea bream egg hatching and larval survival success in control seawater

	Control Replicates (C: Controls)												
Exposure Time (h)	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**Mean/SD/SE**
0-h	17	17	16	16	15	19	14	19	16	16	16	25	
24-h	12	10	14	13	12	16	10	16	11	16	15	20	13.8±2.99/0.86
24 -h Hatching Success %	71	59	88	81	80	84	71	84	69	100	94	80	
48-h	14	14	16	14	15	19	14	16	12	16	15	25	15.8±3.35/0.97
48 -h Hatching Success %	82	82	100	88	100	100	100	84	75	100	94	100	
72-h	14	14	16	14	14	18	14	15	11	14	15	24	15.3±3.19/0.92
72 -h Survival Success %	82	82	100	88	93	95	100	79	69	88	94	96	
96-h	13	13	14	14	14	18	14	15	11	11	14	23	14.5±3.23/0.93
96 -h Survival Success %	76	76	88	88	93	95	100	79	69	69	88	92	

SD: standard deviation, SE: standard error.

4. 12. 1. B Survival of Pre-hatched Larvae in Control Seawater

In twenty one control treatments, larval survival ranged from 80-100% at 24-h exposure period. The mortality observed in 24 h was probably because of physical injury caused during transfer of larvae from stock to exposure wells or the overall health of individual larvae. At 48 h, good survival was maintained and only 3-8% decrease in survival was observed in two treatments. By 72 h, percentage survival was maintained with only six treatments showing 4 to 20% decrease in larval survival (Table 4-4). Further reduction in survival rates was noticed at 96 h with the majority of treatments having survival ranging from 70 to 94%, except in only three treatments where 40% decrease in survival was observed. The standardized laboratory conditions were further improved by carefully in transferring of larvae to enhance survival percentage in controls for a better assessment of toxicant exposure effects.

Table 4-4: Survival of sea bream pre-hatched larvae in control seawater

	Control Replicates (C: control)																				
Exposure Time (h)	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C9**	**C10**	**C11**	**C12**	**C13**	**C14**	**C15**	**C16**	**C17**	**C18**	**C19**	**C20**	**C21**	**Mean/ SD/SE**
0-h	10	12	8	9	10	12	10	10	16	17	17	15	11	10	15	15	15	30	30	30	
24-h	10	12	8	9	10	12	10	10	16	17	15	15	11	10	12	13	14	29	27	28	14.4± 6.3/1.4
24 –h Survival Success %	100	100	100	100	100	100	100	100	100	88	100	100	80	93	97	90	93	100	100	100	
48-h	10	12	8	9	10	11	10	10	16	17	15	15	11	10	12	13	14	29	26	28	14.3± 6.3/1.4
48 -h Survival Success%	100	100	100	100	100	92	100	100	100	88	100	100	80	93	97	87	93	100	100	100	
72-h	10	12	8	9	10	11	10	10	15	16	15	14	9	8	12	13	11	28	26	28	13.8± 6.3/1.4
72 -h Survival Success %	100	100	100	100	100	92	100	100	94	88	93	82	80	73	93	87	93	100	100	100	
96-h	7	9	6	7	8	10	7	6	14	16	14	14	7	7	12	11	9	26	26	27	12.2± 6.8/1.5
96-h Survival Success %	70	75	75	78	80	83	70	60	88	94	82	93	64	70	80	73	60	87	87	90	

SD: standard deviation, SE: standard error.

4. 12. 1. C Effect of Exposure to KCO WAF

Since WAF preparation method can influence the composition, and obviously its toxicity, it is largely debated in scientific literature that which method might clearly demonstrate the true nature of WAF's toxic effect. KCO WAF was either prepared by: 1) variable oil loadings, and 2) by single loading with subsequent serial dilutions of prepared WAF. The rationale for investigating two preparation methods in this study was to understand the toxic behavior of KCO WAF employing sea bream eggs and larvae as test organisms, and which method represents the fate of spilled oil in the case of oil spill incident. Either variable oil loadings or serial dilutions have been thought by numerous studies to represent an oil spill scenario.

The effect of KCO WAF on egg hatching success of sea bream was determined upon exposure to WAF prepared by variable loadings of KCO 1-20 g KCO/L seawater (2-40 g KCO/2L seawater) within a test period of 24 to 48 h as indicated in (Table 4-5). In control, 71% of eggs have hatched to larvae in the first 24 h, whereas, exposure to WAF at different concentrations reduced the percentage of eggs hatched (37-65%). The delay in hatching can be attributed to the toxicant present in crude oil, however; hatching success percentages were not proportional to the concentrations of oil loadings and the pattern was irregular. At 48 h, egg hatching in control exposure increased to 98%, and in other WAF concentrations it further increased and ranged from 85-96%.The variable oil loadings investigated, exerted different toxic effects on sea bream eggs; and each oil loading produced distinctive result (Table 4-5). The 48 h LC_{50} calculation of five replicates ranged from 29.6-64.3 g KCO/L seawater. The average 24 h and 48 h LC_{50} values were >20.0 and 44.0 g KCO/L seawater, respectively, i.e., the highest oil loading used in the bioassay and the average 95% confidence intervals for 48 h ranged between 14.0-1.1x10^{07}. Time and concentration effect of variable oil loadings on egg hatching between five replicates was statistically not significant ($p>0.05$). The no observable effect concentration (NOEC) was <1.0 g KCO/L.

Table 4-5: Percentage sea bream 48 h egg hatching success after exposure to variable oil loadings of KCO WAF (g/L) and control

	Kuwait Crude Oil Loadings (g KCO /L seawater)							
Exposure Time (h)	**Control**	**1.0**	**1.6**	**2.7**	**4.5**	**7.5**	**12.5**	**20.0**
0-h Total Eggs	84	81	82	91	98	81	79	88
24-h Total Eggs	60	51	41	43	41	53	44	37
24-h Hatching Success %	71	63	50	37	42	65	56	42
Mean ± SD/SE	12.0± 1.9/0.8	10.2± 3.3/1.5	8.2± 1.6/0.7	6.8± 2.8/1.2	8.2± 4.6/2.1	10.6± 2.2/1.0	8.8± 1.8/0.8	7.4± 2.5/1.1
48-h Total Eggs	82	75	75	77	84	78	72	79
48-h Hatching Success %	98	93	91	85	86	96	91	90
Mean ± SD/SE	16.4± 1.8/0.8	15.0± 2.9/1.3	15.0± 1.2/0.5	15.4± 2.4/1.1	16.8± 4.1/1.9	15.6± 0.5/0.2	14.4± 2.2/1.0	15.8± 2.8/1.2

SD: standard deviation, SE: standard error.
*** Data were pooled from five replicates.**

In a separate experiment, the effect of KCO WAF on the survival of larvae hatched during a prolonged exposureperiod of up to 96 h was conducted using the same oil loadings (1-20g KCO/L seawater). In controls, 100% of larvae survived at 96-h exposure period, and after exposure to WAF of 1 g KCO/L seawater loading, the survival was the highest (59%); followed by 1.6 and 4.5 g KCO/L seawater oil loadings which were 52% and 50% survival, respectively. Other oil loadings such as 7.5, 20, 12.5 and 2.7 g KCO/L seawater demonstrated lower survival rate 30, 22, 18 and 9 %, respectively (Table 4-6). The average calculated 72 and 96 h LC_{50} value for two replicates were 32.89 and 1.975 g KCO/L seawater, respectively with average 96 h 95% confidence intervals that ranged from 1.176-3.318. Time and concentration effect of variable loading on larval survival during was statistically not significant ($p>0.05$). The NOEC was <1.0 g KCO/L.

Table 4-6: Sea bream egg hatching and larval survival success after exposure to variable oil loadings (1-20 g KCO/L seawater) of KCO WAF and control

	Concentrations (g KCO /L seawater)							
Exposure Time (h)	**Control**	**1.0**	**1.6**	**2.7**	**4.5**	**7.5**	**12.5**	**20**
0-h Total Eggs	28	32	33	32	32	33	34	37
24-h Total Eggs	28	16	15	14	19	20	20	16
24-h Egg Hatching Success %	100	50	45	44	59	61	59	43
Mean ± SD/SE	14.0± 0.0/0.0	8.0± 4.2/3.0	7.5± 2.1/1.5	7.0± 4.2/3.0	9.5± 3.5/2.5	10.0± 4.2/3.0	10.0 ± 2.8/2.0	8.0± 4.2/3.0
48-h Total	28	29	31	29	32	31	33	34
48-h Egg Hatching Success %	100	91	94	91	100	94	97	92
Mean ± SD/SE	14.0± 0.0/0.0	14.5± 4.9/3.5	15.5± 0.7/0.5	14.5± 3.5/2.5	16.0± 0.0/0.0	15.5± 0.7/0.5	16.5± 2.1/1.5	17.0± 1.4/1.0
72-h Total larvae	28	24	29	26	31	24	26	31
72-h Larval Survival Success %	100	75	88	81	97	73	76	84
Mean ± SD/SE	14.0± 0.0/0.0	12.0± 2.8/2.0	14.5± 0.7/0.5	13.0± 1.4/1.0	15.5± 0.7/0.5	12.0± 1.4/1.0	13.0± 0.0/0.0	15.5± 2.1/1.5
96-h Total larvae	28	19	17	3	16	10	6	8
96-h Larval Survival Success %	100	59	52	9	50	30	18	22
Mean ± SD/SE	14.0± 0.0/0.0	9.5± 6.4/4.5	8.5± 4.9/3.5	1.5± 2.1/1.5	8.0± 4.2/3.0	5.0± 1.4/1.0	3.0± 1.4/1.0	4.0± 4.2/3.0

SD: standard deviation, SE: standard error.
***Data were pooled from two replicates.**

In the present study, the toxic effect of KCO WAF prepared by varying oil loadings and serial dilutions was examined. Keeping the water content constant, the effect of variable oil loadings which were subsequently serially diluted indicated variable toxicity responses of sea bream larvae to 1-80 g KCO/L seawater (2-160 g KCO/2L seawater) in order to determine if partitioning of water –accommodated components is increased in the water phase, and whether that in turn increases the toxicity (Table 4-7).

Exposure to WAF of 1 g KCO/L seawater KCO oil loading resulted in survival ranging from 95-99% at the 24 h exposure period. In control treatment, larval survival was 100% at 24 h which decreased to 83% up to 96 h, and in other WAF concentrations it ranged from 95-97% at 24 h which decreased to 23-46% at 96 h with a relatively linear pattern as increasing concentrations yielded lower survival rates. The averaged (LC_{50} g KCO/L ± standard deviation, SD/standard error, SE) values of 9 replicates were 24 h LC_{50} 57.901±102.977.0/34.326.0, 48 h LC_{50} 2.443±1.608/0.536, 72 h LC_{50} 0.36±0.07/0.023 and 96 h 0.12±0.088/0.029 with 96 h 95% confidence intervals of

(0.077-0.187). The combined effects of time and the concentration of 1 g KCO/L loading on fish larval survival was statically significant ($p<0.05$). The NOEC was <0.0625 g KCO/L.

In another experiment, sea bream larvae were exposed to WAF prepared at 10 g KCO/L seawater loading with subsequent 50% dilutions and survival success was followed up to 96-h test period. The percentage of larvae survived in control seawater was 100% at 24 h which decreased to 90% at 96 h, whereas on exposure to KCO WAF (10 g KCO/L) at various dilution concentrations, survival ranged from 93-100% at 24 h, which was reduced to 13-87% at 96 h indicating concentration effect linearity at 25 to 100% concentrations only. The average (LC_{50} g KCO/L ± standard deviation, SD/standard error, SE) concentrations for three replicates obtained with WAF of KCO serial dilution were 24 h LC_{50} 16.841±6.126/3.537, 48 h LC_{50} 14.367±2.326/1.343, 72 h LC_{50} 8.121±2.209/1.275 and 96 h LC_{50} 4.679±3.061/1.767 with 95% confidence intervals of (3.383-6.671) for 96 h, which was less toxic than what was obtained for 1g KCO/L (Table 4-7). Time and concentration combined effect on fish larval survival during exposure to 10 g oil/L seawater was statically significant ($p< 0.05$). The NOEC was <0.0625 g KCO/L.

The effect of the WAF of 20 g KCO/L seawater loading on the survival of sea bream larvae was determined after serial dilution along with seawater controls. In control treatment, larval survival was 100% at 24 h and was reduced to 75% at 96 h; and in other WAF concentrations survival ranged between 93-100% at 24h. At 96 h, larval survival was nonlinear with increasing dilution concentrations, as it ranged from 43-70%, with 0% survival at the highest concentration (Table 4-7) and the only survival linearity was observed at 72 h of exposure. Averaged (LC_{50} g KCO/L ± standard deviation, SD/standard error, SE) concentrations for three replicates were 24 h LC_{50} 77.351±94.747/54.702, 48 h LC_{50} 55.315±27.333/15.781, 72 h LC_{50} 13.955±1.709/0.987 and 96 h LC_{50} 5.761±4.914/2.837 with 95% confidence intervals of (4.467-7.533) for 96 h. Time and concentration combined effect on fish larval survival during exposure to 20 g KCO/L seawater was statically significant ($p< 0.05$). The NOEC was <0.0625 g KCO/L.

The effect of 40 g KCO/L seawater KCO WAF loading on sea bream larval survival indicated that in control treatment, survival was 100% at 24 h exposure period, which decreased to 83% at 96 h. In WAF concentrations, survival was maintained around 90-92% at 72 h and the highest exposure concentration had a 73% reduction in

survival rate. At 96 h, the highest exposure concentration had a 94% reduction in larval survival (Table 4-7). Averaged (LC_{50} g KCO/L ± standard deviation, SD/standard error, SE) concentrations of three replicates were 24 h LC_{50} 61.785±22.197/12.815, 48 h LC_{50} 77.587±33.665/19.436,72 h LC_{50} 32.953±7.529/4.347 and 96 h LC_{50} 11.045±1.867/1.078 g KCO/L with 95% confidence intervals (7.051-17.443) for 96 h. Time and concentration combined effect on fish larval survival during exposure to 40 g KCO/L seawater was statistically significant ($p< 0.05$). The NOEC was <0.0625 g KCO/L.

In this test, the effect of 80 g KCO/L seawater KCO WAF loading demonstrated that the larval survival in control treatment was 100% at 24 h which was reduced to 78% at 96 h. In WAF exposure concentrations, survival ranged between 93-100% at 24 h which decreased to 40-50% at 96 h in lower concentrations with 100% mortality at the highest exposure concentration (Table 4-7). Averaged (LC_{50} g KCO/L ± standard deviation, SD/standard error, SE) concentrations of three replicates were 24 h LC_{50} 120.9±61.003/35.220, 48 h LC_{50} 178.960±81.939/47.308, 72 h LC_{50} 43.981±12.293/7.098 and 96 h LC_{50} 20.533±21.696/1.526 g KCO/L with (16.301-27.069) 95% confidence intervals for 96 h. The combined effect of time and concentration on fish larval survival during exposure to 80g oil/l seawater was statistically significant ($p< 0.05$). The NOEC was <0.0625 g KCO/L.

Table 4-7: Sea bream 96 h larval survival success after exposure to (1-80g oil/L seawater) KCO WAF with serial dilution and control

	Concentration (%)					
Exposure Time (h)	**Control**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
1 g KCO/L seawater-KCO WAF						
0-h	110.0	107.0	102.0	100.0	103.0	105.0
24-h	110.0	103.0	97.0	96.0	98.0	102.0
24-h Survival %	100.0	96.0	95.0	96.0	95.0	97.0
Mean ± SD/SE	12.2± 1.8/0.6	11.4± 2.9 /1.0	10.8± 3.2/1.1	10.7± 2.5/0.8	10.9± 2.1/0.7	11.3± 2.6/0.9
48-h	108.0	90.0	86.0	65.0	77.0	77.0
48-h Survival %	98.0	84.0	84.0	65.0	75.0	73.0
Mean ± SD/SE	12.0± 1.9/0.6	10.0± 3.3/1.1	9.6± 3.4/1.1	7.2± 0.7/0.2	8.6± 1.7/0.6	8.6± 2.4/0.8
72-h	106.0	74.0	68.0	47.0	51.0	35.0
72-h Survival %	96.0	69.0	67.0	47.0	50.0	33.0
Mean ± SD/SE	11.8± 1.5/0.5	8.2± 4.4/1.5	7.6± 3.3/1.1	5.2± 1.9/0.6	5.8± 1.9/0.6	3.9± 3.4/1.1
96-h	91.0	47.0	47.0	27.0	33.0	24.0
96-h Survival %	83.0	44.0	46.0	27.0	33.0	23.0
Mean ± SD/SE	10.1± 2.3/0.8	5.2± 4.2/1.4	5.2± 3.5/1.2	3.0± 1.9/0.6	3.8± 2.5/0.8	2.7± 3.1/1.0
10 g KCO/L seawater-KCO WAF						
0-h	30.0	30.0	30.0	31.0	30.0	30.0
24-h	30.0	28.0	29.0	28.0	30.0	29.0
24-h Survival %	100.0	93.0	97.0	90.0	100.0	97.0
Mean ± SD/SE	10.0± 0.0/0.0	9.3± 1.5/0.9	9.7± 0.6/0.3	9.3± 1.2/0.7	10.0± 1.0/0.6	9.7± 1.5/0.9
48-h	30.0	30.0	28.0	28.0	29.0	29.0
48-h Survival %	100.0	87.0	93.0	90.0	97.0	97.0
Mean ± SD/SE	10.0± 0.0/0.0	8.7± 1.5/0.9	9.3± 0.6/0.3	9.3± 1.2/0.7	9.7± 1.5/0.9	9.7± 1.5/0.9
72-h	30.0	30.0	28.0	28.0	27.0	11.0
72-h Survival %	100.0	80.0	93.0	90.0	90.0	37.0
Mean ± SD/SE	10.0± 0.0/0.0	8.0± 1.0/0.6	9.3± 0.6/0.3	9.3± 1.2/0.7	9.0± 2.6/1.5	3.7± 2.5/1.5
96-h	27.0	12.0	26.0	22.0	19.0	4.0
96-h Survival %	90.0	40.0	87.0	71.0	63.0	13.0
Mean ± SD/SE	9.0± 0.0/0.0	4.0± 3.6/2.1	8.7± 0.6/0.3	7.3± 2.1/1.2	6.3± 4.0/2.3	1.3± 1.5/0.9
20 g KCO/L seawater-KCO WAF						
0-h	36.0	30.0	30.0	30.0	30.0	28.0
24-h	36.0	28.0	28.0	30.0	30.0	26.0
24-h Survival %	100.0	93.3	93.3	100.0	100.0	92.9
Mean ± SD/SE	12.0±0. 0/0.0	9.3±1.2/ 0.7	9.3±0.6/ 0.3	10.0±0. 0/0.0	10.0±0. 0/0.0	8.7±0.6 /0.3
48-h	36.0	28.0	27.0	30.0	28.0	23.0
48-h Survival %	100.0	93.3	90.0	100.0	93.3	82.1
Mean ± SD/SE	12.0±0.	9.3±1.2/	9.0±1.0/	10.0±0.	9.3±0.6/	7.7±0.6

	0/.00	0.7	0.6	0/0.0	0.3	/0.3
72-h	36.0	28.0	26.0	29.0	25.0	1.0
72-h Survival %	100.0	93.3	86.7	96.7	83.3	3.6
Mean ± SD/SE	12.0±0.0/0.0	9.3±1.2/0.7	8.7±1.2/0.7	9.7±0.6/0.3	8.3±2.1/1.2	0.3±0.6/0.3
96-h	27.0	14.0	13.0	21.0	16.0	0.0
96-h Survival %	75.0	46.7	43.3	70.0	53.3	0.0
Mean ± SD/SE	9.0±0.0/0.0	4.7±4.7/2.7	4.3±2.5/1.5	7.0±1.7/1.0	5.3±2.3/1.3	0.0±0.0/0.0
40 g KCO/L seawater-KCO WAF						
0-h	36.0	30.0	30.0	30.0	34.0	31.0
24-h	36.0	30.0	29.0	28.0	33.0	28.0
24-h Survival %	100.0	97.0	97.0	93.0	97.0	90.0
Mean ± SD/SE	12.0±0.0/0.0	9.7±0.6/0.3	9.7±0.6/0.3	9.3±1.2/0.7	11.0±1.0/0.6	9.3±0.6/0.3
48-h	33.0	24.0	27.0	28.0	31.0	27.0
48-h Survival %	92.0	80.0	90.0	93.0	91.0	87.0
Mean ± SD/SE	11.0±0.0/0.0	8.0±2.6/1.5	9.0±0.0/0.0	9.3±1.2/0.7	10.3±1.5/0.9	9.0±1.0/0.6
72-h	33.0	22.0	27.0	27.0	31.0	8.0
72-h Survival %	92.0	73.0	90.0	90.0	91.0	26.0
Mean ± SD/SE	11.0±0.0/0.0	7.3±3.8/2.2	9.0±0.0/0.0	9.0±1.0/0.6	10.3±1.5/0.9	2.7±2.3/1.3
96-h	30.0	16.0	20.0	18.0	21.0	2.0
96-h Survival %	83.0	53.0	67.0	60.0	62.0	6.0
Mean ± SD/SE	10.0±0.0/0.0	5.3±5.0/2.9	6.7±2.5/1.5	6.0±1.0/0.6	7.0±1.0/0.6	0.7±1.2/0.7
80 g KCO/L seawater-KCO WAF						
0-h	27.0	30.0	31.0	30.0	32.0	30.0
24-h	27.0	28.0	30.0	29.0	31.0	30.0
24-h Survival %	100.0	93.0	97.0	97.0	97.0	100.0
Mean ± SD/SE	9.0±0.0/0.0	9.3±1.2/0.7	10.0±1.0/0.6	9.7±0.6/0.3	10.3±0.6/0.3	10.0±0.0/0.0
48-h	27.0	26.0	26.0	28.0	30.0	29.0
48-h Survival %	100.0	87.0	84.0	93.0	94.0	97.0
Mean ± SD/SE	9.0±0.0/0.0	9.0±1.2/0.7	9.0±0.6/0.3	9.3±0.6/0.3	10.0±1.0/0.6	9.7±0.6/0.3
72-h	27.0	24.0	23.0	25.0	25.0	2.0
72-h Survival %	9.0±0.0/0.0	8.0±1.7/1.0	7.7±0.6/0.3	8.3±1.2/0.7	8.3±4.0/2.2	0.7±1.2/0.7
Mean ± SD/SE	100.0	80.0	74.0	83.0	78.0	7.0
96-h	21.0	12.0	13.0	15.0	21.0	0.0
96-h Survival %	78.0	40.0	42.0	50.0	66.0	0.0
Mean ± SD/SE	7.0±0.0/0.0	4.0±3.0/1.7	4.3±0.6/0.3	5.0±3.6/2.1	7.0±4.0/2.3	0.0±0.0/0.0

SD: standard deviation, SE: standard error.

***Data for 1 g/L oil loadings were pooled from nine replicates and for >1 g/L were pooled from three replicates.**

When LC_{50} values of WAF prepared at different oil loadings were compared, it was observed that the most toxic WAF was found to be the one prepared at lowest oil loading, i.e., 1 g KCO/L seawater. WAF prepared with increasing oil loading was not found to exert increasing toxic effects, indicating that saturation of water soluble compounds was achieved at 1g oil/L seawater loadings, and further increase in oil content could not substantially increase partitioning of water soluble compounds in the aqueous medium. This was obvious by chemical analysis of WAF that shows increase in oil loading could slightly increase TPH in WAF which was not proportional to increase in oil loadings and the change in BTEX was even less significant. Nevertheless, if we calculate LC_{50} values on the basis of oil loadings, the toxicity seems to decrease with increasing oil loaded on the water because the oil loaded for WAF preparations was high. This is reflected by the high LC_{50} values which indicates lower toxicity and if the LC_{50} values are low it indicates more toxicity. This uncertainty was removed when the LC_{50} values were equated in terms of TPH in the WAF as reported in Table 4-8; the values were very close at all the WAF preparations made at different oil loadings. This further substantiated our contention that after saturation at about 1 g KCO/L seawater in our preparation vessel further increase in compounds partitioned in water phase was not observed and that is why the LC_{50} value remained more or less same in different exposure of prepared WAF.

Table 4-8: 96-h LC_{50} values expressed on the basis of oil loadings and TPH in WAF determined by two methods

Oil Loading (g KCO/L seawater)	TPH by FT-IR (mg KCO /L seawater)	TPH by Fluorescence (mg KCO /L seawater)	96 LC_{50} equated by oil loading (g KCO /L seawater)±SD/SE	96 LC_{50} equated by TPH FT-IR (g KCO /L seawater)	96 LC_{50} equated by TPH Fluorescence (g KCO /L seawater)
1	2.22	0.308	0.120±0.088/0.029	0.0003	0.00004
10	3.44	0.499	4.679±3.061/1.767	0.0016	0.00023
20	4.77	0.772	5.761±4.914/2.837	0.0014	0.00022
40	7.21	0.781	11.045±1.867/1.078	0.002	0.00022
80	5.78	0.785	20.533±21.696/1.526	0.0015	0.0002

SD: standard deviation, SE: standard error.

To summarize the effect of varying oil loadings, its toxicities were compared against all sea bream life stages such as: egg (E), larvae hatched during exposure (LHE),

and pre-hatched larvae (PHL), it appeared that 1 g KCO/L (PHL) was the most toxic test chemical against sea bream with 96 h LC_{50} of 0.12 g KCO/L, followed by 1.975 g KCO/L for 1-20 g KCO/l (LHE) which was of close toxicity to 1 g KCO/L (PHL), 4.679 g KCO/L for 10 g KCO/L (PHL), 5.761 g KCO/L for 20 g KCO/l (PHL), 11.045 g KCO/L for 40 g KCO/L (PHL), 43.938 g KCO/L 1-20 g KCO/L (E), and 20.533 g KCO/L for 80 g KCO/L (PHL) as the least toxic test chemical against sea bream fish.

4. 12. 1. D Effect of Direct Exposure of Eggs and Larvae to Oil Dispersant

Oil dispersant Corexit® 9527 alone was directly tested as a preliminary tests to investigate and assess its toxicity against sea bream eggs and larvae using six serial dilution of stock solution (0.078 g Corexit® 9527/L seawater).

The effect of Corexit® 9527 on embryonated eggs was examined as ten embryonated eggs were placed in each treatment wells in a triplicate along with controls. Embryonated eggs in control treatments demonstrated 100% hatching into larvae, while in dispersant treatments 100% mortality was found (unsuccessful hatching) in 0.039 and 0.078 g Corexit® 9527/L treatments (Table 4-9). At lower exposure concentrations hatching percentage was 90, 70, 97% for 0.0195, 0.098, 0.0049 g Corexit® 9527/L seawater, respectively. At 48 h of exposure, survival of hatched larvae was decreased from 90 to 3% at 0.0195 g Corexit® 9527/L, whereas at 0.098 g Corexit® 9527/L survival was mildly reduced to 63%, and at 0.0049 g Corexit® 9527/L seawater decreased to 90%, and The 48 h LC_{50} was >0.07 g Corexit® 9527/L seawater. At 72-h of exposure, a noticeable decrease in the survival of larvae in control treatments was also observed, and the percentage dropped from 97% in 48 h to 67%. Exposure to Corexit® 9527 at 0.0195 g Corexit® 9527/L led to the death of all larvae at 72 h. Some survival was observed at 72 h in a sense that at 0.098 g Corexit® 9527/l, it was reduced to 13%, and at 0.0049 g Corexit® 9527/l seawater it was 47%. At the end of test period (96 h), in control 53% of larvae survived and in 0.098 and 0.0049 g/L Corexit® 9527 loadings, 13 % and 37% of larvae survived, respectively (Table 4-9). The 96 h LC_{50} for larvae hatched during exposure was > 0.08 g Corexit® 9527/L seawater. The combined effect of exposure concentration and time on sea bream embryonated egg hatching after exposure to variable loadings of Corexit® 9527 oil dispersant was statistically not

significant ($p > 0.05$), and only exposure time had an effect on egg hatching which was statistically significant ($p < 0.05$). The NOEC was 0.0049 g KCO/L.

The effect of Corexit® 9527 dispersant on sea bream pre-hatched larvae was examined as pre-hatched larvae demonstrated similar effects as that previously observed with embryonated eggs when exposed to similar concentrations of Corexit® 9527 oil dispersant. Pre-hatched larvae exhibited a better survival success than what was achieved with the larvae which have hatched during exposure period. Thus, survival success at 96 h for control, 0.0049 and 0.0098 g Corexit® 9527/L exposures were 50, 67, and 30% respectively (Table 4-10). In higher exposure concentrations, 100% mortality was observed by 96-h exposure period and the 96 h $LC_{50} > 0.08$ g Corexit® 9527/L seawater. The combined effect of exposure concentration and time on sea bream pre-hatched larval survival after exposure to variable loadings of Corexit® 9527 oil dispersant was statistically not significant ($p > 0.05$), and only exposure time had an effect on egg hatching which was statistically significant ($p < 0.05$). The NOEC was 0.0098 g KCO/L.

The LC_{50} values obtained demonstrated close results for the three life stages investigated which was 48 h $LC_{50} > 0.07$ g Corexit® 9527/L seawater for embryonated egg stage, and 96 h LC50 > 0.08 g Corexit® 9527/L seawater for larvae hatched during exposure, and pre-hatched larvae, respectively.

Table 4-9: Hatching success of sea bream eggs after exposure to variable loadings (g dispersant/L seawater) of WAF of Corexit® 9527 dispersant and control

	Concentrations (g Corexit® 9527/L seawater)					
Exposure Time (h)	**Control**	**0.0049**	**0.0098**	**0.0195**	**0.039**	**0.078**
0-h Total Eggs	30.0	30.0	30.0	30.0	30.0	30.0
24-h Total Hatched Eggs	30.0	29.0	21.0	27.0	0.0	0.0
24-h Hatching Success %	100.0	97.0	70.0	90.0	0.0	0.0
Mean ± SD/SE	10.0± 0.0/0.0	9.7± 0.6/0.3	7.0± 1.7/1.0	9.0± 1.7/1.0	0.0± 0.0/0.0	0.0± 0.0/0.0
48-h Total Hatched Eggs	29.0	27.0	19.0	1.0	0.0	0.0
48-h Hatching Success %	97.0	90.0	63.0	3.0	0.0	0.0
Mean ± SD/SE	9.7± 0.6/0.3	9.0± 1.0/0.6	6.3± 1.5/0.9	0.3± 0.6/0.3	0.0± 0.0/0.0	0.0± 0.0/0.0
72-h Total Larvae	20.0	14.0	4.0	0.0	0.0	0.0
72-h Survival Success %	67.0	47.0	13.0	0.0	0.0	0.0
Mean ± SD/SE	6.6± 1.5/0.8	4.7± 3.8/2.2	1.0± 1.4/0.8	0.0± 0.0/0.0	0.0± 0.0/0.0	0.0± 0.0/0.0
96-h Total Larvae	16.0	11.0	4.0	0.0	0.0	0.0
96-h Survival Success%	53.0	37.0	13.0	0.0	0.0	0.0
Mean ± SD/SE	5.3± 1.2/0.7	3.7± 4.6/2.7	1.3± 1.2/0.7	0.0± 0.0/0.0	0.0±0. 0/0.0	0.0± 0.0/0.0

SD: standard deviation, SE: standard error.
*** Data were pooled from three replicates.**

Table 4-10: Survival success of pre-hatched sea bream larvae exposed to Corexit® 9527 dispersant (g dispersant/L seawater) WAF and control

	Concentration (g Corexit® 9527/L seawater)					
Exposure Time (h)	**Control**	**0.0049**	**0.0098**	**0.0195**	**0.039**	**0.078**
0-h Total Larvae	30.0	30.0	30.0	30.0	30.0	30.0
24-h Total Larvae	30.0	30.0	28.0	27.0	0.0	0.0
24-h Survival Success %	100.0	100.0	93.0	90.0	0.0	0.0
Mean ±SD/SE	10.0± 0.0/0.0	10.0± 0.0/0.0	9.3± 1.2/0.7	8.0± 1.7/1.0	0.0± 0.0/0.0	0.0± 0.0/0.0
48-h Total Larvae	27.0	29.0	21.0	9.0	0.0	0.0
48-h Survival Success %	90.0	97.0	70.0	30.0	0.0	0.0
Mean ±SD/SE	9.0± 1.0/0.6	9.7± 0.6/0.3	7.0± 1.7/1.0	3.0± 2.0/1.2	0.0± 0.0/0.0	0.0± 0.0/0.0
72-h Total Larvae	22.0	29.0	11.0	1.0	0.0	0.0
72-h Survival Success %	73.0	97.0	37.0	3.0	0.0	0.0
Mean ±SD/SE	7.3± 1.5/0.9	9.7± 0.6/0.3	3.7± 3.8/2.2	0.3± 0.6/0.3	0.0± 0.0/0.0	0.0± 0.0/0.0
96-h Total Larvae	15.0	20.0	9.0	0.0	0.0	0.0
96-h Survival Success %	50.0	67.0	30.0	0.0	0.0	0.0
Mean ±SD/SE	5.0± 2.0/1.2	6.7± 2.1/1.2	3.0± 3.6/2.1	0.0± 0.0/0.0	0.0± 0.0/0.0	0.0± 0.0/0.0

SD: standard deviation, SE: standard error.
*** Data were pooled from three replicates.**

When Corexit® 9527 CE-WAF was prepared by differential oil loadings of KCO and oil dispersant Corexi[t]® 9527 at 10:1 (oil: dispersant) ratio. The concentrations of KCO were, 0.062, 0.125, 0.25, 0.50 and 1.0 g KCO/L seawater and Corexi[t]® 9527 oil dispersant was added on top of Kuwait crude oil at 0.0062, 0.012, 0.025, 0.050 and 0.10 g Corexi[t]® 9527/L seawater, respectively and WAF was prepared as described in materials and methods. The exposure test was carried for 72 h.

The effect of Corexit® 9527 CE-WAF on embryonated eggs was examined and this showed that, at a 24-h exposure period, 97% of eggs in control treatments successfully hatched and larval survival was reduced to 73%. In the highest exposure concentration of Corexit® 9527 CE-WAF (1 g KCO/L) none of the eggs hatched and at 0.5 g KCO/L CE-WAF exposure, only 3% of the eggs hatched. In CE-WAF prepared at lower concentrations such as 0.25, 0.125, and 0.062 g KCO/L, hatching percentages were 100, 93 and 93 %, respectively. At 48-h exposure period, the two highest concentrations 0.5 and 1.0 g KCO/L exhibited no success in egg hatching and larval survival. A drastic decrease from 100% to 37% was observed at exposure to 0.25g/L concentration (Table 4-11). The other two lower exposure concentrations (0.125 and 0.0625 g KCO/L) showed a reduction in survival percentage comparable to the control. At 72-h exposure period, at 0.25 g KCO/L exposure concentration 100% death of hatched larvae was observed; nevertheless, at other low concentrations 0.125 and 0.0625 g KCO/L, 47 and 60% larvae survived (Table 4-11). The calculated 48 h LC_{50} for egg hatching and 72 h LC_{50} value for larvae hatched during exposure were > 1.0 g KCO/L seawater. The combined effect of exposure concentration and time on sea bream egg hatching after exposure to CE-WAF of Corexit® 9527 oil dispersant was statistically not significant ($p > 0.05$), and the combined effect of exposure concentration and time on the survival of sea bream larvae hatched during exposure to CE-WAF of Corexit® 9527 oil dispersant was statistically not significant ($p > 0.05$), and only the exposure time had a significant effect on larval survival ($p<0.05$). The NOEC was < 0.062 g KCO/l and The effect of Corexit® 9527 CE-WAF on pre-hatched larvae indicated that in control treatment, survival of sea bream pre-hatched larvae was 97% at 24 h which was reduced to 87% at 72 h.

The response of pre-hatched sea bream larvae was similar to that observed with embryonated eggs to CE-WAF since exposure at 0.5 and 1 g KCO/L loading caused 100% mortality at 24 h. At 0.25 g KCO/l exposure concentration, 97% of larvae survived at 24-h exposure period, but all larvae at 48 h died. At lower concentrations,

0.125 and 0.062 g KCO/L, 97% of larvae survived at 24-h exposure period and the survival decreased to 80% at 48 h and 33% at 72 h (Table 4-12). The calculated LC_{50} 72 h LC_{50} value for pre-larvae hatched was > 1.0 g KCO/L. It can be concluded is that higher CE-WAF concentrations caused 100% mortality in sobaity embryonated eggs and larvae. The combined effect of exposure concentration and time on the survival of sobaity pre-hatched larvae after exposure to CE-WAF of Corexit® 9527 oil dispersant was statistically not significant ($p > 0.05$), and only the exposure time had a significant effect on larval survival ($p<0.05$). The NOEC was < 0.062 g KCO/L.

Table 4-11: Sea bream egg hatching and larval survival success after exposure to CE- WAF of KCO dispersed with Corexit® 9527 and control

		Concentration (g KCO/g Corexit® 9527dispersant/L seawater)				
Exposure Time (h)	**Control**	**0.062 g + 0.0062 g**	**0.125 g + 0.0125 g**	**0.25 g + 0.025 g**	**0.5 g + 0.05 g**	**1.0 g + 0.1 g**
0-h Total Eggs	30	30	30	30	30	30
24-h Total Eggs	29	28	28	30	1	0
24-h Hatching Success %	97%	93%	93%	100%	3%	0%
Mean ±SD/SE	9.7±0.6/ 0.3	8.7±1.5/ 0.9	9.3±1.2/ 0.7	10.0±0.0/ 0.0	0.3±0.6/ 0.3	0.0±0.0/ 0.0
48-h Total Egg	28	26	27	11	0	0
48-h Hatching Success %	93%	87%	90%	37%	0%	0%
Mean ±SD/SE	9.3±0.6/0.3	8.7±1.5/ 0.9	9.0±1.7/ 1.0	3.7±1.2/ 0.7	0.0±0.0/ 0.0	0.0±0.0/ 0.0
72-h Total Larvae	22	14	18	0	0	0
72-h Survival Success %	73%	47%	60%	0%	0%	0%
Mean ±SD/SE	7.3±1.2/0.7	4.7±/4.5/ 2.6	6.0±1.7/ 1.0	0.0±0.0/ 0.0	0.0±0.0/ 0.0	0.0±0.0/ 0.0

SD: standard deviation, SE: standard error.
*** Data were pooled from three replicates.**

Table 4-12: Sea bream pre-hatched larval survival success after exposure to CE-WAF of KCO dispersed with Corexit® 9527 Dispersant and control

		Concentration (g KCO/g Corexit® 9527dispersant/L seawater)				
Exposure Time (h)	**Control**	**0.062 g + 0.0062 g**	**0.125 g + 0.0125 g**	**0.25 g + 0.025 g**	**0.5 g + 0.05 g**	**1.0 g + 0.1 g**
0-h Total Larvae	30	30	30	30	30	30
24-h Total Larvae	29	29	29	29	0	0
24-h Survival Success %	97%	97%	97%	97%	0%	0%
24-h Mean ±SD/SE	9.7±0.6/0.3	9.7±0.6/ 0.3	9.7±0.6/ 0.3	9.3±0.6/ 0.3	0.0±0.0/ 0.0	0.0±0.0/ 0.0
48-h Total Larvae	29	24	24	0	0	0
48-h Survival Success %	97%	80%	80%	0%	0%	0%
48-h Mean ±SD/SE	9.7±0.6/0.3	8.0±1.0/ 0.6	8.0±1.0/ 0.6	0.0±0.0/ 0.0	0.0±0.0/ 0.0	0.0±0.0/ 0.0
72-h Total Larvae	26	10	10	0	0	0
72-h Survival Success %	87%	33%	33%	0%	0%	0%
72-h Mean ±SD/SE	8.7±0.6/0.3	3.3±1.5/ 0.9	3.3±1.5/ 0.9	0.0±0.0/ 0.0	0.0±0.0/ 0.0	0.0±0.0/ 0.0

SD: standard deviation, SE: standard error.
*** Data were pooled from three replicates.**

Comparison of the effect of two preparation methods, such as the variable oil loadings method and single loading with serial dilution method was conducted by using WAF of KCO dispersed by Corexit® 9527 at 10:1 (oil: dispersant) ratio in the following manner: 0.0062, 0.012, 0.025, 0.050 and 0.10 g of Corexit® 9527 dispersant layered over 0.062, 0.125, 0.25, 0.50 and 1.0 g of KCO per liter seawater, respectively. In addition, a stock solution of KCO WAF single loading at 1.0 g KCO and 0.1 g Corexist® 9527 was prepared and serially diluted (50% dilution) to the concentration equivalent to the oil loading concentrations. Total petroleum hydrocarbons (TPH) (mg/L) concentrations were estimated in CE-WAF prepared by variable oil loading and by a single loading which was serially diluted. TPH concentrations in CE-WAF prepared from dispersed crude oil by variable oil loadings were not linear to the measured oil loadings (Table 4-13). Conversely, when CE-WAF solution was prepared using single oil loading and subsequent dilutions resulted in more linear TPH concentrations. Depending on the TPH linearity observed in the variable dilution method concentration, response relationship can be better workout (Table 4-13).

At the 72-h exposure period, the only surviving larvae were noticed in the lowest exposure concentration (0.062 g KCO + 0.0062 g dispersant/L seawater) of both preparation methods. No surviving larvae were noticed at higher exposure concentrations (Table 4-14).

The calculated 48 h LC_{50} for sea bream egg hatching and, larvae hatched during exposure to variable loadings of KCO CE-WAF using Corexit® 9527 dispersant was the same (>1.0 g KCO/L) and the effect of exposure time and concentration using variable oil loading on egg hatching success was statistically slightly significant ($p= 0.06$), but it was statistically not significant on larvae hatched during exposure ($p>0.05$) and only exposure time was statistically significant ($p<0.05$). The NOEC was < 0.062 g KCO/L. Similarly, the 72 h LC_{50} for pre-hatched larvae was >1.0 g KCO/L and the effect of exposure time and concentration using variable oil loading on pre-hatched larval survival success was statistically not significant ($p>0.05$) and only exposure time was statistically significant ($p<0.05$). The NOEC was < 0.062 g KCO/L.

Sea bream eggs exposed to serial dilutions of single loading of KCO CE-WAF using Corexit® 9527 dispersant appeared to be more sensitive to the CE-WAF toxicity compared to the toxicity of variable loadings method (Table 4-14). The egg stage had a 48 h LC_{50} of 0.268 g KCO/l seawater (95% confidence intervals of 0.250-0.287) which indicated more resistance to toxicity to what was observed for larvae hatched during

exposure which were more sensitive with 0.69 g KCO/L seawater as a 72 h LC_{50} with 61.4-73 as 95% confidence intervals. The effect of exposure time and concentration using variable dilution (serial dilutions) on egg hatching success was statistically significant ($p<0.05$), but it was statistically not significant on larvae hatched during exposure ($p>0.05$) and only exposure time had a significant effect ($P<0.05$). The NOEC was 0.625 g KCO/L.

Pre-hatched larvae exposed to CE-WAF serial dilutions was even more sensitive to CE-WAF toxicity than larvae hatched during exposure with 0.06 g KCO/L as a 72 h LC_{50} with 0.057-0.063 as 95% confidence intervals. The effect of exposure time and concentration using variable dilution (serial dilutions) on pre-hatched larval survival success was statistically not significant ($p>0.05$) and only exposure time had a significant effect ($P<0.05$). The NOEC was < 0.625 g KCO/L.

Table 4-13: Comparison of TPH concentrations (mg/L) by variable oil/dispersant loadings and serial dilution methods

CE-WAF Loading KCO g + Corexit® 9527 g + L seawater		**CE-WAF Serial Dilution of 1 g KCO + 0.1 g Corexit® 9527 + 1 L seawater**	
KCO g/ Dispersant g/ L Seawater	**TPH Concentration (mg/L)**	**Concentration (%)**	**TPH Concentration (mg/L)**
0.0625 g + 0.00625 g + L SW	1.2	6.25%	1.5
0.125 g + 0.0125 g + L SW	7.4	12.5%	3.0
0.25 g + 0.025 g + L SW	11.0	25%	5.3
0.5 g + 0.05 g + L SW	15.3	50%	9.1
1 g + 0.1 g + L SW	16.0	100%	17.2

Table 4-14: Comparison of KCO CE-WAF prepared by variable oil loadings and by serial dilutions of single loading and Corexit® 9527 on three sea bream life stages

				Variable Loading Concentration (KCO g/ Dispersant g/L seawater)				
			Control	**0.062 g + 0.0062g + L SW**	**0.125 g + 0.0125 g + L SW**	**0.25 g + 0.025 g + L SW**	**0.5 g + 0.05 g + L SW**	**1.0g + 0.1g + L SW**
			Variable Dilution Concentration (%)					
Preparation Method	**Life Stage**	**Exposure Time(h)**	**Control**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
Variable Loading	Egg	0-h	30	30	30	30	30	30
	Egg	24-h	29	28	28	30	1	0
	Egg	48-h	28	26	27	11	0	0
	Larvae	72-h	22	14	18	0	0	0
72-h Survival %			73%	47%	60%	0%	0%	0%
Serial Dilutions*	Egg	0-h	30	30	30	30	30	30
	Egg	24-h	29	29	30	26	26	0
	Egg	48-h	28	26	24	17	0	0
	Larvae	72-h	22	17	0	0	0	0
72-h Survival %			73%	57%	0%	0%	0%	0%
Variable Loading	Pre-hatched Larvae	0-h	30	30	30	30	30	30
	Pre-hatched Larvae	24-h	29	29	29	29	0	0
	Pre-hatched Larvae	48-h	29	24	24	0	0	0
	Pre-hatched Larvae	72-h	26	10	10	0	0	0
72-h Survival %			87%	33%	33%	0%	0%	0%
Serial Dilutions*	Pre-hatched Larvae	0-h	30	30	30	30	30	30
	Pre-hatched Larvae	24-h	30	30	30	29	0	0
	Pre-hatched Larvae	48-h	29	13	0	0	0	0
	Pre-hatched Larvae	72-h	26	9	0	0	0	0
72-h Survival %			87%	30%	0%	0%	0%	0%

Note: SW stands for seawater.

4. 12. 1. E Effect of KCO CE-WAF Prepared by Other Dispersant Products

The above experiment has shown that serial dilution of WAF prepared at fixed oil loadings produced better linearity in the distribution of TPH in WAF; therefore this method was used for comparing the effects of all the three dispersants chosen for this study. CE-WAFs were prepared using single oil loading of (10:1) oil to dispersant ratio which is equivalent to 1 g KCO/L seawater and 0.1 g dispersants and its toxicity was determined against sea bream pre-hatched larvae.

The effect of CE-WAF of KCO using Corexit® 9500 dispersant on larval survival success for three replicates is recorded in Table 4-15 and at 96-h exposure period, survival percentage was 75% in control compared to survival in CE-WAF exposure which resulted in total mortality at the highest exposure concentration (100%). At lower exposure concentrations, 50% and below, higher survival percentages were observed, and a good linearity between increasing exposure concentration and larval mortality rates was observed (Table 4-15). The addition of Corexit® 9500 oil dispersant, have not increased KCO WAF toxicity, hence the value of Corexit® 9500 LC_{50} is higher than the KCO WAF LC_{50} indicating less toxicity. The average (LC_{50} g/L ± standard deviation, SD/standard error, SE) concentrations indicate that the 24 h LC_{50} was 4.118±1.597/9.22, the 48 h LC_{50} was 0.881±0.125/0.072, the 72 h LC_{50} was 0.682±0.134/0.077.0, and the 96 h LC_{50} was 0.291±0.027/0.0153; with 0.207-0.410 95% confidence intervals for 96 h only. The combined effect of Corexit® 9500 CE-WAF exposure concentration and time on sobaity pre-hatched larval survival was statistically significant ($p<0.05$). The NOEC was 0.25 g KCO/L.

Effect of CE-WAF of KCO using Corexit® 9527 dispersant on larval survival success for three replicates demonstrated that in control treatment, at 96-h survival percentage was 75% compared to survival in CE-WAF exposure, which resulted in total mortality at higher exposure concentrations (25, 50, and 100%) (Table 4-15). The average (LC_{50} g/L ± standard deviation, SD/standard error, SE) concentrations indicate that the 24 h LC_{50} was 0.345±0.012/0.007, and its toxicity have increased with increasing exposure period, so the 48 h LC_{50} was 0.198±0.046/0.027, the 72 h LC_{50} was 0.150±0.0062/0.004, and the 96-h LC_{50} 0.121±0.011/0.0064. In comparison to what was achieved for Corexit® 9500 CE-WAF (0.291 g KCO/L seawater), KCO WAF alone (0.120 g KCO/L seawater), clearly Corexit® 9527 CE-WAF was more toxic to sea bream larvae than other treatments.

The combined effect of exposure concentration and time on sobaity pre-hatched larval survival was statistically not significant ($p>0.05$). The NOEC was 0.0625 g KCO/L.

Effect of CE-WAF of KCO using Slickgone® dispersant on larval survival success of sobaity larvae for three replicates was similar to that of Corexit® 9500 CE-WAF with total mortality in the highest exposure concentration (100%). In control treatment survival was 89% up till 96 h, and in lower exposure concentrations, survival percentages were more proportional to concentrations (Table 4-15). The average (LC_{50} g/L ± standard deviation, SD/standard error, SE) concentrations indicate that the estimated 24 h LC_{50} was 1.0±0.0/0.0 which decreased with increasing exposure period so the 48 h LC_{50} values were 0.711±0.098/0.056, 72 h LC_{50} 0.628±0.058/0.033, and 96 h LC_{50} 0.426±0.121/0.07. The LC_{50} values clearly demonstrated that there was a gradual increase in the toxicity of Slickgone® CE-WAF for the first three days of the bioassay, but 96 h its toxicity was less than that of all CE-WAFs, and KCO WAF solution alone. The combined effect of exposure concentration and time on sobaity pre-hatched larval survival was statistically significant ($p<0.05$). The NOEC was 0.25 g KCO/L.

Table 4-15: Toxicity effect of three CE-WAF solutions prepared by single loading and serial dilutions method on sea bream pre-hatched larvae and control

		Concentrations (%)					
Chemical Name	**Exposure Time (h)**	**Control**	**6.25%**	**12.50%**	**25%**	**50%**	**100%**
	0-h	36.0	33.0	33.0	31.0	32.0	30.0
	24-h	36.0	33.0	33.0	30.0	31.0	29.0
	24-h Survival %	100.0%	100.0%	100.0%	97.0%	97.0%	97.0%
	Mean±SD /SE	12.0±0.0 /0.0	11.0±1.7 /1.0	11.0±0.0 /0.0	10.0±1.0 /0.6	10.3±1.5 /0.9	9.7±0.6 /0.3
	48-h	36.0	33.0	33.0	30.0	31.0	5.0
Corexit® 9500	48-h Survival %	100.0%	100.0%	100.0%	97.0%	97.0%	17.0%
	Mean±SD /SE	12.0±0.0 /0.0	11.0±1.7 /1.0	11.0±0.0 /0.0	10.0±1.0 /0.6	10.3±1.5 /0.9	1.7±1.5 /0.9
CE-WAF	72-h	36.0	32.0	33.0	30.0	28.0	1.0
	72-h Survival %	100.0%	97.0%	100.0%	97.0%	88.0%	3.0%
	Mean±SD /SE	12.0±0.0 /0.0	11.0±2.1 /1.2	11.0±0.0 /0.0	10.0±1.0 /0.6	9.3±2.1/ 1.2	0.3±0.6 /0.3
	96-h	27.0	28.0	27.0	28.0	15.0	0.0
	96-h Survival %	75.0%	85.0%	82.0%	90.0%	47.0%	0.0%
	Mean±SD /SE	9.0±0.0/ 0.0	9.3±2.0/ 0.9	9.0±2.0/ 1.0	9.3±0.6/ 0.3	5.0±4.0/ 2.0	0.0±0.0 /0.0
	0-h	36.0	33.0	31.0	29.0	34.0	31.0
	24-h	36.0	32.0	31.0	29.0	0.0	0.0
	24-h Survival %	100.0%	97.0%	100.0%	100.0%	0.0%	0.0%
Corexit® 9527	Mean±SD /SE	12.0±0.0 /0.0	11.0±1.2 /0.7	10.3±2.0 /0.9	10.0±0.6 /0.3	0.0±0.0/ 0.0	0.0±0.0 /0.0
	48-h	36.0	31.0	29.0	6.0	0.0	0.0
CE-WAF	48-h Survival %	100.0%	94.0%	94.0%	21.0%	0.0%	0.0%
	Mean±SD /SE	12.0±0.0 /0.0	10.3±0.6 /0.3	10.0±2.0 /0.9	2.0±3.0/ 2.0	0.0±0.0/ 0.0	0.0±0.0 /0.0
	72-h	36.0	31.0	27.0	0.0	0.0	0.0
	72-h Survival %	100.0%	94.0%	87.0%	0.0%	0.0%	0.0%
	Mean±SD /SE	12.0±0.0 /0.0	10.3±0.6 /0.3	9.0±1.0/ 0.6.0	0.0±0.0/ 0.0.	0.0±0.0/ 0.0	0.0±0.0 /0.0
	96-h	27.0	25.0	15.0	0.0	0.0	0.0
	96-h Survival %	75.0%	76.0%	48.0%	0.0%	0.0%	0.0%
	Mean±SD /SE	9.0±0.0/ 0.0	8.3±0.6/ 0.3	5.0±1.0/ 0.6	0.0±0.0/ 0.0	0.0±0.0/ 0.0	0.0±0.0 /0.0
	0-h	46.0	43.0	43.0	41.0	41.0	42.0
Slickgone® NS	24-h	46.0	41.0	43.0	41.0	41.0	42.0
	24-h Survival %	100.0%	95.0%	100.0%	100.0%	100.0%	100.0%
CE-WAF	Mean±SD	12.0±0.0	10.0±0.6	11.3±2.3	11.0±2.0	11.0±1.2	11.0±1.

/SE	/0.0	/0.3	/1.3	/1.0	/0.7	2/0.7
48-h	44.0	37.0	40.0	39.0	29.0	4.0
48-h Survival %	96.0%	86.0%	93.0%	95.0%	71.0%	10.0%
Mean±SD	12.0±0.0	10.0±0.6	11.0±2.0	11.0±1.2	10.3±0.6	0.7±1.2
/SE	/0.0	/0.3	/1.0	/0.7	/0.3	/0.7
72-h	44.0	37.0	40.0	37.0	8.0	0.0
72-h Survival %	96.0%	86.0%	93.0%	90.0%	20.0%	0.0%
Mean±SD	12.0±0.0	10.0±0.6	11.0±2.0	11.0±1.2	10.0±2.0	0.0±0.0
/SE	/0.0	/0.3	/1.0	/1.0	1.0	/0.0
96-h	41.0	35.0	40.0	35.0	5.0	0.0
96-h Survival %	89.0%	81.0%	93.0%	85.0%	12.0%	0.0%
Mean±SD /SE	9.0±0.0/ 0.0	10.0±1.0 /0.3	11.0±2.0 /1.0	10.3±2.0 /1.0	7.0±2.1/ 1.2	0.0±0.0 /0.0

SD: standard deviation, SE: standard error.
***Data for individual chemicals were pooled from three replicates.**

4. 12. 1. F Developmental Abnormalities in Sea Bream Larvae After Exposure to KCO WAF

Only 1 g KCO/L seawater KCO WAF loading was selected for developmental abnormalities in sea bream larvae. Larval survival ranged from 93 to 100% at 24-h exposure period. As exposure period was increased, 20-30% reduction in survival was witnessed at 72 h and 40-60% reduction at 96 h was observed (Table 4-16). The average (LC_{50} g/L ± standard deviation, SD/standard error, SE) concentrations for three replicates indicated that the estimated 24 h LC_{50} was 6.267±8.192/4.730, the 48 h LC_{50} values were 7.545±8.238/4.756, 72 h LC_{50} 1.109±0.466/0.269, and 96 h LC_{50} 0.083±0.017/0.01.

In controls, no deformities were observed in any larvae assayed for 96 h. After exposure of sobaity larvae to 1g KCO/L loading, five deformity types were observed during 96 h bioassay in all exposure concentrations. In all the dilution series total deformity percentages indicated that Type-A deformity (59%) was the most common deformity type in all dilutions, followed by Type-B deformity (16%), Type-C (15%), Type-E (10%), then Type-D (3%). As the exposure concentrations increased, the deformity percentages increased from 9% at 6.25% concentration to 31% at 100% exposure concentration (Table 4-17). Other deformity types were encountered too at lower percentages (Plate 4-2).

Table 4-16: Survival Success of Sobaity Larvae after Exposure to (1 g/L) KCO WAF and Control

	Concentration (%)					
Exposure Time (h)	**Control**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
0-h	48.0	33.0	30.0	34.0	31.0	29.0
24-h	48.0	33.0	28.0	33.0	29.0	29.0
24-h Survival %	100.0	100.0	93.0	97.0	94.0	100.0
24-h Mean± SD/SE	16.0± 0.0/0.0	11.0± 3.0/2.0	9.3± 1.2/1.0	11.0± 2.0/1.0	10.0± 2.0/1.0	10.0± 1.0/0.3
48-h	48.0	31.0	24.0	33.0	29.0	27.0
48-h Survival %	100.0	94.0	80.0	97.0	94.0	93.0
48-h Mean± SD/SE	16.0± 0.0/0.0	10.3± 2.0/1.0	8.0± 1.0/1.0	11.0± 2.0/1.0	10.0± 2.0/1.0	9.0± 1.0/1.0
72-h	45.0	26.0	18.0	27.0	25.0	19.0
72-h Survival %	94.0	79.0	60.0	79.0	81.0	66.0
72-h Mean± SD/SE	15.0± 0.0/0.0	9.0± 1.2/1.0	6.0± 2.0/1.2	9.0± 4.0/2.1	8.3± 1.2/1.0	6.3± 1.2/1.0
96-h	42.0	9.0	9.0	16.0	12.0	8.0
96-h Survival %	88.0	27.0	30.0	47.0	39.0	28.0
96-h Mean± SD/SE	14.0± 0.0/0.0	3.0± 1.0/0.6	3.0± 2.0/1.0	5.3± 2.0/1.0	4.0± 1.0/1.0	3.0± 1.2/1.0

SD: standard deviation, SE: standard error.

***Data for individual chemicals were pooled from three replicates.**

Table 4-17: Percentages of deformity types of sobaity larvae after exposure to 1 g KCO/L and control

		Percentages of Deformity Types (%)					
Concentration (%)	**Number of Exposed Larvae**	**A**	**B**	**C**	**D**	**E**	**Total Percentages (%)**
Control	48	0.0	0.0	0.0	0.0	0.0	0.0
6.25%	33	3.0	0.0	6.0	0.0	0.0	9.0
12.5%	30	13.0	3.0	3.0	0.0	0.0	19.0
25%	34	12.0	0.0	6.0	3.0	0.0	21.0
50%	31	10.0	3.0	0.0	0.0	10.0	23.0
100%	29	21.0	10.0	0.0	0.0	0.0	31.0

Notes: The numbers indicate the percentages of larvae deformed. (0.0%) indicates no deformity of any type. Other deformity types are denoted with letters like: A- Lordosis (V-Shaped, inward curvature of the spine), B-Kyphosis (hunch back), C-Scoliosis (lateral bending of the spine), D-Irregular body shape, E- Deformed caudal fin.

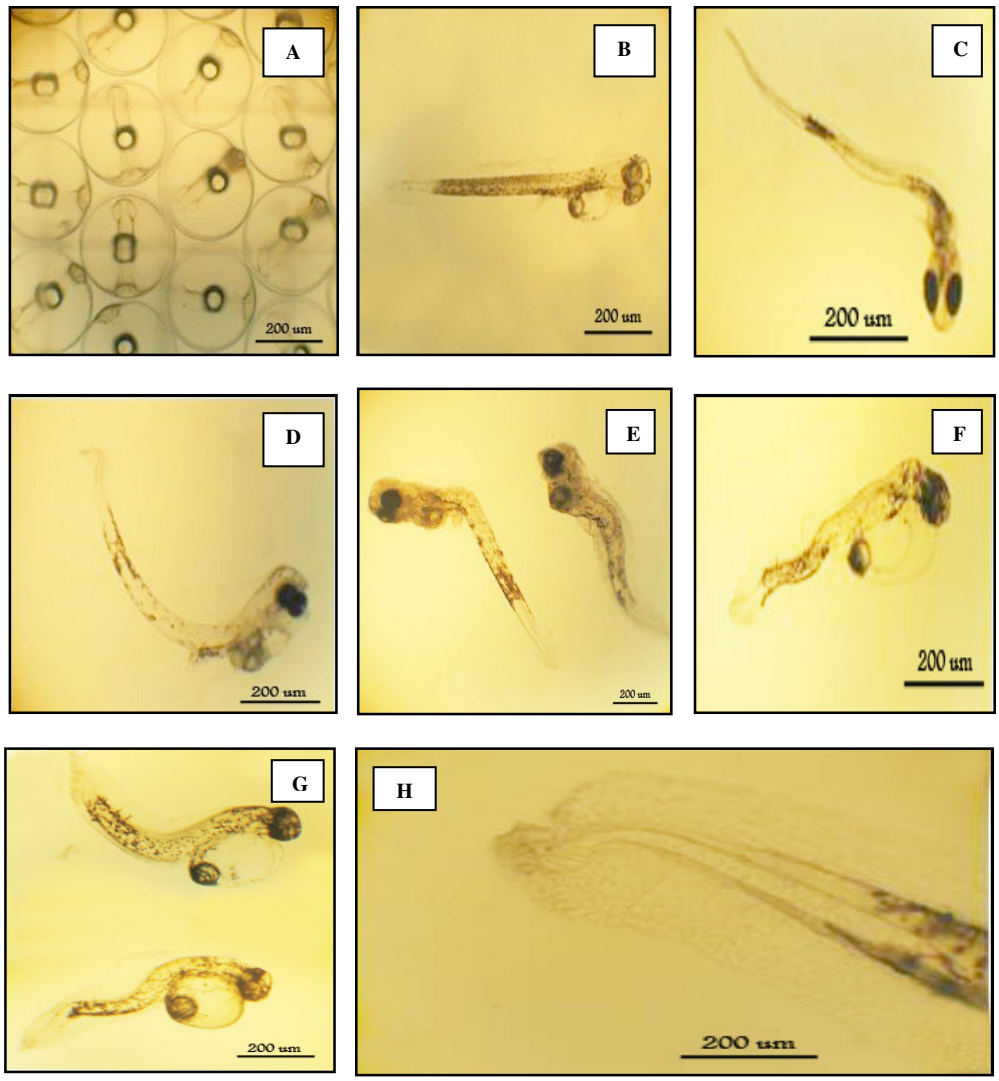


Plate 4-2: (A) healthy control sea bream embryonated egg, (B) healthy control larvae, (C) scoliosis (lateral-bending of spine), (D) lordosis (V-shaped inward curvature of spine), (E) kyphosis (hunchback), (F) irregular body shape, (G) yolk sac edema, and (H) deformed caudal fin.

4. 12. 2 Hamoor-(Orange-Spotted Grouper)

4. 12. 2. A Exposure to Control Seawater

Average hatching success of hamoor eggs in 15 control treatments at 24 h was 96% with the most of control treatments having more than 90% hatching. Some eggs which didn't hatch at 24 h eventually hatched at 48h, and 100% hatching was achieved (Table 4-18). Hamoor larvae which hatched during control exposure demonstrated a 97% survival at 96 h period with only 7-10% of larval mortality at the end of exposure period.

Table 4-18: Hamoor egg hatching and larval survival success in control seawater

	Control Replicates (C: Controls)															
Exposure Time (h)	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	**C14**	**C15**	**Mean±SD/SE**
0-h	16	20	16	16	15	15	19	18	16	17	10	10	10	10	11	15.0±4.0/0.9
24-h	16	18	16	15	14	15	19	18	16	16	8	10	10	9	11	14.1±4.0/0.9
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	15.0±4.0/0.9
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	15.0±4.0/0.9
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	14.3±4.0/1.0
96-h Survival Success %	100	100	100	100	100	93	100	100	100	100	100	90	90	90	91	

SD: standard deviation, SE: standard error.

4. 12. 2. B Effect of KCO WAF

Hamoor eggs exposed to 1 g oil/l seawater (2 g KCO/2l seawater) KCO WAF showed above 90% hatching in most of exposure concentrations at 24 h exposure period, similar to that found in controls. Hatching percentages were increased to 100% at most of the concentrations except at highest concentration where it decreased to 87% at 48 h exposure period. Eggs which didn't hatch at either 24 or 48 h were considered dead beyond this point (Table 4-19). The average (48 h LC_{50} g oil/L seawater ± standard deviation, DS/standard error, SE) calculated of three replicates was >1.0±0.0/0.0 with a 95% confidence interval of (0.933-1.072) for 48 h. The effect of exposure time and concentration on hamoor egg hatching was statistically not significant ($p>0.05$), and only exposure concentration had a statistically significant effect ($p<0.05$). The NOEC was <1.0 g KCO/L.

Hamoor larvae which hatched during exposure showed 100% larval survival up till 96-h of exposure in controls, and at 3.12, 6.25, and 12.5% dilutions of WAF of KCO. The survival of larvae was reduced by 2% and 53% at 25, 50% KCO WAF dilutions, respectively. At the highest exposure concentration, 13% of the eggs were not hatched, and the larvae which hatched could not survive and all died by 96-h of exposure (Table 4-19). The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of three replicates were 24 h LC_{50} 1.035±0.06/0.035, 48 h LC_{50} 1.0±0.0/0.0, 72 h LC_{50} 0.92±0.21/0.12, and 96 h LC_{50} 0.46±0.1/0.06 with a 95% confidence interval of (0.321-0.752) for 96 h LC_{50}. The effect of exposure time and concentration on hamoor egg hatching was statistically significant ($p<0.05$). The NOEC was 0.25 g KCO/L.

Hamoor pre-hatched larvae were exposed to serially diluted KCO WAF 1 g KCO/ seawater (2 g KCO/2L seawater). In controls, survival success was 97% for 24-h exposure period which decreased to 96% at 96 h, and the 100% concentration of KCO WAF, exerted some toxic effect as survival percentage was reduced from 100% at 24-h exposure period to 21% at 96 h. At lower dilutions, minimal effect was observed as survival ranged from 87 to 99% up till 96-h exposure period (Table 4-20). The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of nine replicates were 24 h LC_{50} was 1.26 g KCO/L seawater for pre-hatched larvae which was of comparable toxicity to both 24 and 48h LC_{50} for egg stages which were 1.075 and >1.0 g KCO/L seawater, respectively. Conversely, the average LC_{50} values of nine

replicates for pre-hatched larvae were 24 h LC_{50} 1.26±0.56/0.19, 48 h LC_{50} 1.25±0.3/0.10, 72 h LC_{50} 1.47±1.55/0.52, and 96 h LC_{50} 0.93±0.77/0.25 seawater with (0.465-0.917) 95% confidence intervals for 96 h, which demonstrated more resistance (less toxicity) to WAF of KCO than larvae hatched during KCO WAF exposure (96 h LC_{50} 0.468 g KCO/L seawater). The effect of exposure time and concentration of 1g KCO/L seawater on hamoor larval survival success was statistically significant ($p<0.05$). The NOEC was <0.25 g KCO/L.

The effect of serially diluted 20 g oil/L seawater (40 g oil/2L seawater) KCO loading on hamoor larval survival success is recorded in (Table 4-21), as survival in control seawater was successful (100%) up to 96-h exposure period. Survival percentages in exposure concentration ranged between 98-100% at 24 h, and survival in 100% concentration decreased from 100 to 0% at 96-h exposure. Survival percentages in other lower concentrations ranged between 33-75% at 96 h. The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of four replicates were: 24 h LC_{50} 34.0±40.0/20.0, 48 h LC_{50} 31.0±11.0/5.3, 72 h LC_{50} 25.0±12.0/6.0, and 96 h LC_{50} 6.0±1.0/0.4. The LC_{50} values for 1 g KCO/L seawater KCO loading was (0.93 g KCO/L seawater) of nine replicates, which appeared to be more toxic compared to 20 g oil/L seawater KCO loading with an 96 h LC_{50} value of (6.0 g KCO/L seawater) done for four replicates with (3.95-8.42) confidence intervals for 96 h. The effect of exposure time and concentration of 20 g KCO/L seawater on hamoor larval survival success was statistically significant ($p<0.05$). The NOEC was <0.077 g KCO/L.

It appeared that 1 g oil/L seawater KCO loading had the ability to be more readily partitioned in seawater than 20 g KCO/L seawater loading during KCO WAF preparation, which contributed more to its toxicity against hamor larvae, taking into consideration preparation vessel dimensions, mixing duration, and etc.

Table 4-19: Hamoor egg and larval survival success after exposure to 1 g KCO/L seawater and control

	Concentrations (%)						
Exposure Time (h)	**Control**	**3.12%**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
0-h Total Egg	45.0	45.0	44.0	47.0	43.0	47.0	52.0
24-h Total Egg	45.0	43.0	44.0	46.0	43.0	42.0	50.0
24-h Egg Hatching Success %	100.0	96.0	100.0	98.0	100.0	89.0	96.0
24-h Mean±SD/SE	15.0± 0.0/0.0	14.3± 1.0/0.3	15.0± 1.0/0.3	15.3± 1.0/ 0.3	14.3± 2.0/1.0	14.0± 1.0/ 1.0	17.0± 8.1 /5.0
48-h Total Egg	45.0	45.0	44.0	47.0	43.0	47.0	45.0
48-h Larval Survival Success %	100.0	100.0	100.0	100.0	100.0	100.0	87.0
48-h Mean±SD/SE	15.0± 0.0/0.0	15.0± 2.0/1.0	15.0± 1.0/0.3	16.0±1.0/ 0.3	14.3± 2.0/1.0	16.0± 1.2/ 1.0	15.0± 10.0/6.0
Total Larvae	45.0	45.0	44.0	47.0	43.0	46.0	14.0
72-h Larval Survival Success %	100.0	100.0	100.0	100.0	100.0	98.0	27.0
72-h Mean±SD/SE	15.0± 0.0/0.0	15.0± 2.0/1.0	15.0± 1.0/0.3	16.0±1.0/ 0.3	14.3± 2.0/1.0	15.3± 2.0/ 1.0	5.0± 5.0/3.0
Total Larvae	45.0	45.0	44.0	47.0	42.0	22.0	0.0
96-h Larval Survival Success %	100.0	100.0	100.0	100.0	98.0	47.0	0.0
96-h Mean±SD/SE	15.0± 0.0/0.0	15.0± 2.0/1.0	15.0± 1.0/0.3	16.0± 1.0/ 0.3	14.0± 2.0/1.0	7.3± 8.0/ 5.0	0.0± 0.0/0.0

SD: standard deviation, SE: standard error.

***Data were pooled from three replicates.**

Table 4-20: Survival success of hamoor pre-hatched larvae after exposure 1 g KCO/L seawater KCO WAF and control

	Concentration (%)					
Exposure Time (h)	**Control**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
0-h	90.0	90.0	90.0	90.0	90.0	90.0
24-h	86.0	89.0	89.0	90.0	90.0	90.0
24-h Survival Success %	97.0	99.0	99.0	100.0	100.0	100.0
Mean±SD/SE	10.0±1.0/ 0.3	10.0±0.3/ 0.1	10.0±0.3/ 0.1	10.0±0.0 /0.0	10.0±0.0/ 0.0	10.0±0.0/ 0.0
48-h	87.0	89.0	89.0	90.0	88.0	79.0
48-h Survival Success %	97.0	99.0	99.0	100.0	98.0	88.0
Mean±SD/SE	10.0±1.0/ 0.3	10.0±0.3/ 0.1	10.0±0.3/ 0.1	10.0±0.0 /0.0	10.0±0.4/ 0.1	9.0±2.0/ 1.0
72-h	87.0	89.0	89.0	87.0	88.0	37.0
72-h Survival Success %	97.0	99.0	99.0	97.0	98.0	41.0
Mean±SD/SE	9.0±1.1/ 0.4	10.0±0.3/ 0.1	10.0±0.3/ 0.1	10.0±1.0 /0.2	10.0±1.0/ 0.2	4.1±4.0/ 1.2
96-h	86.0	89.0	89.0	85.0	78.0	19.0
96-h Survival Success %	96.0	99.0	99.0	94.0	87.0	21.0
Mean±SD/SE	9.3±1.1/ 0.4	10.0±0.4/ 0.1	10.0±0.3/ 0.1	9.4±1.0/ 0.2	9.0±1.4/0. 0	2.1±3.0/ 1.0

SD: standard deviation, SE: standard error.

***Data were pooled from nine replicates.**

Table 4-21: Survival success of hamoor pre-hatched larvae after exposure to (20 g KCO/L seawater) of KCO WAF and control

	Concentration %						
Exposure Time (h)	**Control**	**7.7%**	**12.9%**	**21%**	**36%**	**60%**	**100%**
0-h Total Larvae	40.0	41.0	41.0	36.0	39.0	39.0	37.0
24-h Total Larvae	40.0	41.0	40.0	36.0	39.0	39.0	37.0
24-h Survival Success %	100.0	100.0	98.0	100.0	100.0	100.0	100.0
Mean±SD/SE	10.0±0.0/ 0.0	10.3±1.0/ 0.3	10.0±1.0/ 0.4	9.0±1.4/ 1.0	10.0±1.0/ 0.3	10.0±1.0/ 0.3	9.3±1.0/ 1.0
48-h Total Larvae	40.0	41.0	40.0	36.0	38.0	37.0	26.0
48-h Survival Success %	100.0	100.0	98.0	100.0	97.0	95.0	70.0
Mean±SD/SE	10.0±0.0/ 0.0	10.3±1.0/ 0.3	10.0±1.0/ 0.4	9.0±1.4/ 1.0	10.0±1.0/ 0.3	9.3±1.0/ 0.3	7.0±2.0/ 1.0
72-h Total Larvae	40.0	39.0	37.0	35.0	37.0	35.0	14.0
72-h Survival Success %	100.0	95.0	90.0	97.0	95.0	90.0	38.0
Mean±SD/SE	10.0±0.0/ 0.0	10.0±1.0/ 0.3	9.3±1.0/ 0.5	9.0±2.0/ 1.0	9.3±1.0/ 0.3	9.0±1.0/ 0.3	4.0±2.4/ 1.2
96-h Total Larvae	40.0	29.0	27.0	27.0	27.0	13.0	0.0
96-h Survival Success %	100.0	71.0	66.0	75.0	69.0	33.0	0.0
Mean±SD/SE	10.0±0.0/ 0.0	7.3±2.2/ 1.1	7.0±1.0/ 1.0	7.0±2.0/ 1.0	7.0±2.1/ 1.0	3.3±2.0/ 1.0	0.0±0.0/ 0.0

SD: standard deviation, SE: standard error.
***Data were pooled from four replicates.**

4. 12. 2. C Effect of Direct Exposure to KCO CE-WAF

The effect of Corexit® 9500 CE-WAF on hamoor embryonated eggs was examined and it revealed that, in controls, 100% egg hatched at 24-h exposure period; while in KCO CE-WAF exposure, at most concentrations 91-98% hatching was observed. However, by 48 h, there was a 20-30% reduction of egg hatching in most CE-WAF concentrations except at 12.5% were it remained the same, and at 100% concentration, a drastic 80% decrease of hatched eggs was noticed (Table 4-22). The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of five replicates were for 24 h LC_{50} was 1.811±0.46/0.21 and for 48 h LC_{50} was

0.53±0.13/0.06 for the egg stage with 95% confidence intervals ranging from 0.356-1.49 for the 48 h exposure. The effect of exposure time and concentration of Corexit® 9500 CE-WAF on hamoor egg hatching success was statistically significant ($p<0.05$). The NOEC was <0.0625 g KCO/L.

Survival of hamoor larvae which have hatched during exposure, demonstrated 100% survival up to 96-h in control seawater, while in all exposure concentrations, a decrease in survival rates was observed. At 100% CE-WAF exposure concentration, none of the hatched eggs survived up to 96 h. Further reduction of larval survival which has hatched during exposure was observed at lower exposure concentration (Table 4-22). The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of five replicates for 24 h LC_{50} was 2.05±1.3/1.0, 48 h LC_{50} was 0.6±0.2/0.07, 72 h LC_{50} was 0.41±0.1/0.06, and 96 h LC_{50} was 0.21±0.12/0.05 with 95% confidence intervals ranging from 0.15-0.3 for 96 h only. The effect of exposure time and concentration of Corexit® 9500 CE-WAF on hamoor larval survival success was statistically significant ($p<0.05$). The NOEC was <0.0625 g KCO/L.

In the control, survival of hamoor pre-hatched larvae was 94% up to 96-h exposure period, and in other serial dilutions such as 1.56 and 3.12% survival; was 65 and 37% up to 48 h, respectively. 100% mortality was recorded for higher concentrations (6.25 to 50%) indicating high toxicity, taking into consideration that 100% concentration was eliminated since it was shown in preliminary tests that it killed all hamoor pre-hatched larvae (Table 4-23). The only concentration which demonstrated survival at 96 h was 1.56% with 41% survival percentage. The LC_{50} g oil/L seawater calculated were 0.257, 0.028, 0.019, 0.015 g KCO/l seawater for 24, 48, 72, 96 h respectively, with (0.013-0.018) as a 95% confidence interval. The effect of Corexit® 9500 CE-WAF's exposure time and concentration on hamoor pre-hatched larval survival success was statistically not significant ($p>0.05$), and only exposure time caused a significant statistical effect ($p<0.05$). The NOEC was <0.0156 g KCO/L.

Table 4-22: Hatching success of hamoor eggs and larval survival after exposure to Corexit® 9500 CE-WAF and control

	Concentrations (%)					
Exposure Time (h)	**Control**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
0 h Total Eggs	55.0	55.0	61.0	56.0	57.0	56.0
24 h Total Eggs	55.0	53.0	60.0	51.0	52.0	51.0
24-h Hatching Success %	100.0	96.0	98.0	91.0	91.0	91.0
24-h Mean±SD/ SE	11.0±2.2/ 1.0	11.0±2.0/ 1.0	12.0±4.0/ 2.0	10.2±0.4/ 0.2	10.4±3.0/ 1.2	10.2±4.4/ 2.0
48 h Total Eggs	55.0	49.0	60.0	36.0	43.0	6.0
48-h Hatching Success %	100.0	89.0	98.0	64.0	75.0	11.0
48-h Mean±SD/ SE	11.0±2.2/ 1.0	10.0±3.0/ 1.2	12.0±4.0/ 2.0	7.0±4.4/ 2.0	9.0±3.2/ 1.4	1.2±1.1/ 0.0
72 h Total Larvae	55.0	48.0	57.0	31.0	37.0	2.0
72-h Survival Success %	100.0	87.0	93.0	55.0	65.0	4.0
72-h Mean±SD/ SE	11.0±2.2/ 1.0	10.0±3.0/ 1.3	11.4±4.3/ 2.0	6.2±4.3/ 2.0	7.4±4.0/ 2.0	0.4±1.0/ 0.2
96 h Total Larvae	55.0	37.0	51.0	25.0	13.0	0.0
96-h Survival Success %	100.0	67.0	84.0	45.0	23.0	0.0
96-h Mean±SD/ SE	11.0±2.2/ 1.0	7.4±5.0/ 2.2	10.2±5.0/ 2.2	5.0±5.0/ 2.1	3.0±2.0/ 1.0	0.0±0.0/ 0.0

Data were pooled from three replicates.
***SD: standard deviation, SE: standard error.**

Table 4-23: Survival success of hamoor pre-hatched larvae after exposure to Corexit® 9500 CE-WAF and control

	Concentration (%)						
Exposure Time (h)	**Control**	**1.56%**	**3.12%**	**6.25%**	**12.5%**	**25%**	**50%**
0-h	32.0	37.0	30.0	26.0	33.0	31.0	32.0
24- h	32.0	33.0	26.0	19.0	23.0	20.0	1.0
24-h Survival Success %	100.0	89.0	87.0	73.0	70.0	65.0	3.0
48- h	31.0	24.0	11.0	0.0	0.0	0.0	0.0
48-h Survival Success %	97.0	65.0	37.0	0.0	0.0	0.0	0.0
72-h	31.0	22.0	0.0	0.0	0.0	0.0	0.0
72-h Survival Success %	97.0	59.0	0.0	0.0	0.0	0.0	0.0
96-h	30	15.0	0.0	0.0	0.0	0.0	0.0
96-h Survival Success %	94	41.0	0.0	0.0	0.0	0.0	0.0

The effect of exposure to Corexit® 9527 CE-WAF indicated that successful egg hatching was achieved at 24-h exposure period in control treatment (100%) and in lower CE-WAF exposure hatching ranged from 85-89% except at 50 and 100% concentrations, hatching percentages were 31 and 0%, respectively (Table 4-24). At 48 h, none of the eggs hatched at 50 and 100% CE-WAF concentrations, whereas at lower concentrations sharp reduction in hatching range was noticed. Further mortality of hatched larvae was observed at 25% and lower CE-WAF exposure. The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of four replicates for 24 h LC_{50} was 0.339±0.178/0.089 and for 48 h LC_{50} was 0.171±0.055/.0.27 with confidence intervals ranging from 0.128-0.229. The effect of exposure time and concentration of Corexit® 9527 CE-WAF on hamor egg hatching success was statistically significant ($p<0.05$). The NOEC was <0.0625 g KCO/L.

Larval survival was 100% in control seawater at 96-h period and 65, 30 and 5% at 6.25, 12.5, and 25% CE-WAF exposure concentrations, respectively. And above those concentrations, 100% mortality was observed which reflected the toxic effect of Corexit® 9527 CE-WAF on hamoor larvae (Table 4-24). The average (LC_{50} g oil/l seawater ± standard deviation, SD / standard error, SE) calculated of four replicates for 24 h LC_{50} was 0.345±0.173/0.086, 48 h LC_{50} 0.164±0.069/0.034, 72 h LC_{50} 0.131±0.022/0.011, and 96 h LC_{50} 0.087±0.020/0.010 with 95% confidence intervals ranging from 0.063-0.121 for 96 h LC_{50} only. The effect of exposure time and concentration of Corexit® 9527 CE-WAF on hamoor larval survival success was statistically not significant ($p>0.05$). The NOEC was <0.0625 g KCO/L.

In control, survival of hamoor pre-hatched larvae was 67% up to 96-h exposure period, and in all exposure concentrations it ranged from 26 to100% at 24 h, which decreased to 100% mortality in all concentrations at 96-h exposure period (Table 4-25). The LC_{50} values for Corexit 9527® CE-WAF exposure were 0.296, 0.014, 0.010 g KCO/L seawater, for 24, 48, 72, respectively, and 0.010 g KCO/l seawater 96 h respectively with (0.089-0.0113) as a 95% confidence interval. The effect of Corexit® 9527 CE-WAF exposure exhibited more toxicity (0.010 g KCO/L seawater) than Corexit® 9500 CE-WAF with a 96-h LC_{50} value of 0.015 g KCO/L seawater and KCO-WAF (0.934 g KCO/L seawater). The effect of Corexit® 9527 CE-WAF's exposure time and concentration on hamoor pre-hatched larval survival success was statistically not significant ($p>0.05$), and only exposure time caused a significant statistical effect ($p<0.05$). The NOEC was <0.0156 g KCO/L and.

Table 4-24: Hatching success of hamoor eggs and larval survival after exposure to Corexit® 9527 CE-WAF and control

	Concentrations (%)					
Exposure Time (h)	**Control**	**6.25%**	**12.50%**	**25%**	**50%**	**100%**
Total Eggs	45.0	46.0	47.0	55.0	45.0	57.0
24 h Total Eggs	45.0	39.0	42.0	48.0	14.0	0.0
24-h Egg Hatching Success %	100.0	85.0	89.0	87.0	31.0	0.0
24-h Mean±SD/SE	11.3±3.0 /1.3	10.0±4.0 /2.0	11.0±3.4 /2.0	12.0±1.4 /0.7	4.0±7.0 /4.0	0.0±0.0 /0.0
48 h Total Eggs	45.0	35.0	31.0	30.0	0.0	0.0
48-h Egg Hatching Success %	100.0	76.0	66.0	55.0	0.0	0.0
48-h Mean±SD/SE	11.3±3.0 /1.3	9.0±4.2 /2.1	8.0±5.1 /3.0	8.0±3.0 / 1.4	0.0±0.0 /0.0	0.0±0.0 /0.0
72 h Total Larvae	45.0	35.0	28.0	11.0	0.0	0.0
72-h Larval Survival Success %	100.0	76.0	60.0	20.0	0.0	0.0
72-h Mean±SD/SE	11.3±3.0 /1.3	9.0±4.2 /2.1	7.0±5.0 /2.3	3.0±3.0 /1.0	0.0±0.0 /0.0	0.0±0.0 /0.0
96 h Total Larvae	45.0	30.0	14.0	3.0	0.0	0.0
96-h Larval Survival Success	100.0	65.0	30.0	5.0	0.0	0.0
96-h Mean±SD/SE	11.3±3.0 /1.3	8.0±5.0 /2.3	4.0±2.1 /1.0	0.8±2.0 /1.0	0.0±0.0 /0.0	0.0±0.0 /0.0

***SD: standard deviation, SE: standard error.**
Data were pooled from five replicates.

Table 4-25: Survival success of hamoor pre-hatched larval after exposure to Corexit® 9527 CE-WAF and control

	Concentration (%)						
Exposure Time (h)	**Control**	**1.56%**	**3.12%**	**6.25%**	**12.5%**	**25%**	**50%**
0-h	43.0	42.0	33.0	38.0	41.0	43.0	39.0
24- h	40.0	42.0	26.0	30.0	28.0	20.0	10.0
24-h Survival Success %	93.0	100.0	79.0	79.0	68.0	47.0	26.0
48- h	36.0	13.0	0.0	0.0	0.0	0.0	0.0
48-h Survival Success %	48.0	31.0	0.0	0.0	0.0	0.0	0.0
72- h	31.0	0.0	0.0	0.0	0.0	0.0	0.0
72-h Survival Success %	72.0	0.0	0.0	0.0	0.0	0.0	0.0
96 -h	29.0	0.0	0.0	0.0	0.0	0.0	0.0
96-h Survival Success %	67.0	0.0	0.0	0.0	0.0	0.0	0.0

The effect of Slickgone® CE-WAF on egg hatching revealed that, in control treatment, 100% egg hatching was achieved at 24-h exposure period, and in CE-WAF exposure; hatching ranged from 91-98% at all the concentrations. By 48h, there was 10-15% decrease in hatching rates with a major decline (36%) at 100% exposure

concentration (Table 4-26). The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of four replicates for 24 h LC_{50} was 3.34±2.55/1.28 and the 48 h LC_{50} was 2.34±1.69/0.85 g KCO/L seawater with (0.453-2.147) 95% confidence intervals. The effect of exposure time and concentration of Slickgone® CE-WAF on hamoor egg hatching success was statistically significant ($p<0.05$). The NOEC was <0.0625 g KCO/L.

In control treatment, 100% of larvae survived at 96-h exposure period. A linear decrease in survival with increasing concentration was achieved at all exposure concentration, in which survival ranged from 83% at 6.25% concentration to 47% at 100% exposure concentration by 96 h (Table 4-26). The average (LC_{50} g oil/L seawater ± standard deviation, SD / standard error, SE) calculated of four replicates for 24 h LC_{50} 8.931±12.944/6.472, 48 h LC_{50} 1.914±1.287/0.644, 72 h LC_{50} 1.548±1.3/0.650, and 96 h LC_{50} 1.185±0.919/0.460 with (0.524-3.236) 95% confidence intervals for 96 h LC_{50} only. The effect of exposure time and concentration of Slickgone® CE-WAF on hamoor larval survival success was statistically significant ($p<0.05$). The NOEC was <0.0625 g KCO/L.

The percentage survival of hamoor pre-hatched larvae in control seawater was 80% at 96 h, and exposure to CE-WAF treatments resulted in total mortality in three higher concentrations (12.5, 25, and 50%) at 96 h. At lower exposure concentrations, survival percentages ranged from 29 to 79% at 96-h exposure period (Table 4-27). The LC_{50} values for Slickgone® CE-WAF were 0.464, 0.0821, 0.0508, and 0.0452 g KCO/L seawater at 24, 48, 72, and with (0.0409-0.0498) 95% confidence intervals, and still it was more toxic than that of KCO-WAF (0.934 g KCO/L seawater). The effect of Slickgone® CE-WAF's exposure time and concentration on hamoor pre-hatched larval survival success was statistically not significant ($p>0.05$), and only exposure time caused a significant statistical effect ($p<0.05$). The NOEC was 0.0315 g KCO/L.

Table 4-26: Hatching success of hamoor eggs and larval survival after exposure to Slickgone® CE-WAF and control

	Concentrations (%)					
Exposure Time (h)	**Control**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
0 h Total Eggs	40.0	40.0	44.0	44.0	42.0	45.0
24 h Total Eggs	40.0	38.0	40.0	41.0	41.0	43.0
24-h Hatching Success %	100.0	95.0	91.0	93.0	98.0	96.0
24-h Mean±SD/SE	10.0±0.0 /0.0	10.0±1.0 0.3	10.0±1.0 /0.4	10.3±1.0 /0.3	10.3±1.0 /0.3	11.0±1.0 /0.0
48 h Total Larvae	40.0	35.0	39.0	38.0	35.0	27.0
48-h Hatching Success %	100.0	88.0	89.0	86.0	83.0	60.0
48-h Mean±SD/SE	10.0±0.0 /0.0	9.0±1.0 /0.0	10.0±1.3 /1.0	10.0±1.3 /1.0	9.0±1.3 /1.0	7.0±5.0 /2.4
72 h Total Larvae	40.0	35.0	39.0	36.0	24.0	22.0
72-h Survival Success %	100.0	88.0	89.0	82.0	57.0	49.0
72-h Mean±SD/SE	10.0±0.0 /0.0	9.0±1.0 /0.0	10.0±1.3 /1.0	9.0±1.4 /1.0	6.0±4.1 /2.0	6.0±4.1 /2.1
96 h Total Larvae	40.0	33.0	33.0	32.0	22.0	21.0
96-h Survival Success %	100.0	83.0	75.0	73.0	52.0	47.0
96-h Mean±SD/SE	10.0±0.0 /0.0	8.3±1.3 /1.0	8.3±2.1 /1.0	8.0±2.0 /1.0	6.0±4.0 /2.0	5.3±4.1 /2.1

***SD: standard deviation, SE: standard error.**
Data were pooled from four replicates.

Table 4-27: Survival success of hamoor pre-hatched larval after exposure to Slickgone® CE-WAF and control

	Concentration (%)						
Exposure Time (h)	**Control**	**1.56%**	**3.12%**	**6.25%**	**12.5%**	**25%**	**50%**
0-h	44.0	39.0	38.0	45.0	47.0	36.0	47.0
24- h	44.0	39.0	38.0	45.0	45.0	31.0	19.0
24-h Survival Success %	100.0	100.0	100.0	100.0	96.0	86.0	40.0
48- h	40.0	39.0	34.0	29.0	5.0	5.0	0.0
48-h Survival Success %	91.0	100.0	89.0	64.0	11.0	14.0	0.0
72- h	40.0	36.0	28.0	17.0	0.0	0.0	0.0
72-h Survival Success %	91.0	92.0	74.0	38.0	0.0	0.0	0.0
96 -h	35.0	31.0	25.0	13.0	0.0	0.0	0.0
96-h Survival Success %	80.0	79.0	66.0	29.0	0.0	0.0	0.0

4. 12. 2. D LC_{50} Results Equated in Terms of TPH Concentrations

When LC_{50} values for sea bream were calculated on the basis of oil loading used for CE-WAF preparation (1 g KCO/L seawater), values were of comparable toxicity for Corexit® 9527 CE-WAF and KCO WAF, but it were less toxic for Corexit® 9500 and

Slickgone® CE-WAF. However, when LC_{50} values were equated using TPH values (analyzed by FT-IR method) Corexit® 9527 and Slickgone® CE-WAF were of comparable toxicity against sea bream and their LC_{50} values were 0.0021 g KCO/L seawater, except Corexit® 9500 was less toxic with an LC_{50} of 0.0097 g KCO/L seawater and KCO WAF was the most toxic with an LC_{50} of 0.0003 g KCO/L seawater. When CE-WAF values were equated using TPH values (analyzed by Fluorescence method); KCO WAF was the most toxic followed by Slickgone® CE-WAF > Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF and their respective LC_{50} were 0.00004, 0.0013, 0.0043, and 0.0069 g KCO/L seawater, respectively (Table 4-28).

For orange-spotted grouper, CE-WAFs were more toxic as compared to KCO WAF, but when LC_{50} values were equated using TPH values (analyzed by FT-IR method) KCO WAF oil loadings, Corexit® 9527 and Slickgone® CE-WAF were of close toxicity values with 0.0003, 0.0002, 0.0002 g KCO/L seawater; respectively and except Corexit® 9500, was less toxic (0.0005 g KCO/L seawater). And when CE-WAF values were equated using TPH values (analyzed by Fluorescence method); KCO WAF and Slickgone® CE-WAF were of comparable toxicity (0.0001 g KCO/L seawater) but more toxic than Corexit® 9527 and Corexit® 9500 CE-WAFs (0.0004 g KCO/l seawater), respectively (Table 4-28).

Although, TPH concentration (FT-IR) were the highest for Corexit® 9500 CE-WAF among other CE-WAFs and WAF, but its toxicity when equated in terms of TPH was the lowest against sea bream, this indicates that TPH is not an accurate method of chemically quantifying WAF and/ or CE-WAF and to date there is no accurate method of doing so. Toxicity results based on oil loadings provide a better understanding of test chemical behavior.

Table 4-28: LC_{50} values for sea bream and orange-spotted grouper equated in terms of TPH concentrations by FT-IR and Fluorescence method (Flu.)

			Fish Type					
	Chemical Analysis		Sobaity - Sea Bream			Hamoor - Orange Spotted Grouper		
Chemical Name	TPH by FT-IR (mg/L)	TPH by Flu. (mg/L)	LC_{50} (g/L) oil loading	LC_{50} by TPH (FT-IR) (g/L)	LC_{50} by TPH (Flu.) (g/L)	LC_{50} (g/L) oil loading	LC_{50} by TPH (FT-IR) (g/L)	LC_{50} by TPH (Flu.) (g/L)
KCO WAF Corexit® 9500	2.22	0.308	0.12	0.0003	0.00004	0.934	0.0003	0.0001
CE-WAF Corexit® 9527	33.2	23.7	0.291	0.0097	0.0069	0.015	0.0005	0.0004
CE-WAF Slickgone®	17.67	35.2	0.121	0.0021	0.0043	0.010	0.0002	0.0004
CE-WAF	5.05	3.0	0.426	0.0021	0.0013	0.045	0.0002	0.0001

4. 12. 3 Shea'am (Yellow-fin Sea Bream)

The effect of KCO WAF on yellow-fin sea bream (*Acanthopagrus latus*) locally named Shea'am was examined. Pre-hatched larvae (24 h old) were exposed to 50% dilution of 20 g KCO/L seawater WAF which was diluted to make six concentrations in a way that each concentration was 50% of the previous one, thus 3.12, 6.25, 12.5, 25, 50, 100% of WAF and a seawater control was used for the bioassay. Survival in the control was 98% at 24 h which decreased to 60% at 96 h. Survival in exposure concentrations at 24 h was greater than 90% then slightly decreased at 96 h and was above 85%; only in the lowest and highest concentrations survival was around 50% (Table 4-29). The LC_{50} values were 47.0, 34.0, 34.0, and 22.0 g KCO/L seawater for 24, 48, 72, and 96-h exposure periods, respectively; with 20.0-25.3 as 95% confidence intervals. The effect of exposure concentration and time of 20 g KCO/l seawater on yellow-fin sea bream pre-hatched larval survival was statistically not significant ($p>0.05$), only exposure time was statistically significant ($p<0.05$). The NOEC was 0.0312 g KCO/L.

Table 4-29: Survival success percentages of yellow-fin sea bream pre-hatched larvae exposed to 20 g oil/L of KCO WAF and control

	Concentration (%)						
Exposure Time (h)	**Control**	**3.12%**	**6.25%**	**12.5%**	**25%**	**50%**	**100%**
0-h	45.0	47.0	48.0	47.0	47.0	47.0	47.0
24-h	44.0	47.0	48.0	47.0	47.0	46.0	44.0
24-h Survival Success %	98.0	100.0	100.0	100.0	100.0	98.0	94.0
48-h	31.0	27.0	47.0	44.0	46.0	45.0	42.0
48-h Survival Success %	69.0	57.0	98.0	94.0	98.0	96.0	89.0
72-h	30.0	26.0	46.0	44.0	46.0	42.0	38.0
72-h Survival Success %	67.0	55.0	96.0	94.0	98.0	89.0	81.0
96-h	27.0	23.0	42.0	40.0	42.0	40.0	25.0
96-h Survival Success %	60.0	49.0	88.0	85.0	89.0	85.0	53.0

4. 12. 4 Meid-(Mullet)

Mullet (*Liza klunzingeri*) 24-h old pre-hatched larvae were exposed to variable oil loadings of KCO WAF prepared in a manner that each concentration is 60% of the preceding one, thus 1, 1.6, 2.7, 4.5, 7.5, 12.5, and 20 g KCO/L seawater which represents (2 to 40 g KCO/2L seawater). Mullet larvae maintained 100% survival at 24 and 48 h in control, but at 72 and 96 h decreased to 90%. Treatment with KCO WAF at different concentrations produced variable and irregular survival success percentages for 24-h exposure period which ranged from 72 to 95%, but at 48-h exposure period, survival percentages decreased by 20-30% in most of the oil loadings. By 96-h exposure period, a 5 to 10% decreases in survival percentages was observed and survival ranged between 21-90% in KCO WAF exposure test and non-linearity was observed between oil loadings and survival percentages (Table 4-
30). The LC_{50} values decreased during the exposure period indicating increasing toxicity and they were 125.0, 13.0, 9.0, and 5.0 g KCO/L seawater for 24, 48, and 72 and 96 h, respectively with (3.88-7.02) as 95% confidence intervals. The effect of exposure concentration and time of 1- to 20 g KCO/L seawater on mullet pre-hatched larval survival was statistically significant ($p<0.05$). The NOEC was 1.6 g KCO/L.

Table 4-30:Survival success of mullet pre-hatched larvae after exposure to variable oil loadings of KCO (g KCO/L seawater) and control

	Concentration (g KCO/L seawater)							
Exposure Time (h)	**Control**	**1.0**	**1.6**	**2.7**	**4.5**	**7.5**	**12.5**	**20.0**
0-h	20.0	21.0	20.0	20.0	18.0	18.0	23.0	24.0
24-h	20.0	20.0	19.0	19.0	13.0	17.0	20.0	22.0
24-h Survival Success %	100.0	95.0	95.0	95.0	72.0	94.0	87.0	92.0
48-h	20.0	16.0	19.0	12.0	9.0	13.0	16.0	12.0
48-h Survival Success%	100.0	76.0	95.0	60.0	50.0	72.0	70.0	50.0
72-h	18.0	15.0	19.0	11.0	7.0	13.0	13.0	5.0
72-h Survival Success%	90.0	71.0	95.0	55.0	39.0	72.0	57.0	21.0
96-h	18.0	15.0	18.0	8.0	5.0	11.0	12.0	5.0
96-h Survival Success %	90.0	71.0	90.0	40.0	28.0	61.0	52.0	21.0

4. 12. 5 Comparison between 72 and 96 h LC_{50} Values

Comparison of 72 and 96 h LC_{50} concentrations obtained after exposure of sea bream larvae to WAF and CE-WAF of KCO revealed that exposure to nine replicates of 1 g KCO/L seawater loading resulted in consistent 72 h LC_{50} concentrations which were in the range of 0.315-0.380 g KCO/L seawater. KCO loadings > 1 g KCO/L seawater (1-80 g KCO/L seawater) generally produced a consistent pattern between the three replicates for individual loadings (Table 4-31). For CE-WAFs of KCO, LC_{50} concentrations obtained for 72 h were consistent between the three replicates same as to what was achieved for 96 h LC_{50} concentrations.

Exposure of orange-spotted grouper larvae to 1 and 20 g KCO/L seawater loading resulted in 72 h LC_{50} concentrations for six out of nine replicates which ranged from 0.621-0.001747 g KCO/L seawater. Similarly, a consistent pattern for nine replicates was achieved for 96 h LC_{50} concentrations which ranged between 0.450-2.973 g KCO/L seawater. LC_{50} concentrations for 72 and 96 h obtained for Corexit® 9500 and 9527 CE-WAFs, were consistent between replicates, however; 72 and 96 h LC_{50} concentrations obtained for Slickgone® CE-WAF were irregular between replicates as indicated in Table 4-32.

Table 4-31: Comparison between 72 and 96 h LC_{50} for sea bream larvae

KCO LC_{50} Comparison										
72 h LC_{50} (g/L)										
Replicates										
KCO (g/L)	**Loading**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1		0.332	0.336	0.38	0.367	0.37	0.315	0.276	0.525	0.336
10		9.573	5.579	9.21						
20		12.106	14.28	15.478						
40		37.928	24.292	36.64						
80		47.472	54.152	30.32						
96 h LC_{50} (g/L)										
Replicates										
KCO (g/L)	**Loading**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1		0.055	0.132	0.101	0.054	0.057	0.135	0.055	0.132	0.101
10		8.0	1.9	4.1						
20		2.12	11.35	3.8						
40		9.1	11.2	12.8						
80		11.1	45.3	5.1						

CE-WAFs LC_{50} Comparison									
LC_{50} (g/L)	**Corexit® 9527**		**CE-WAF**	**Corexit® 9500**		**CE-WAF**	**Slickgone® CE-WAF**		
72 h LC_{50} (g/L)	0.157	0.147	0.145	0.798	0.714	0.535	0.569	0.684	0.63
96 h LC_{50} (g/L)	0.108	0.125	0.129	0.317	0.293	0.264	0.352	0.361	0.566

Table 4-32: Comparison between 72 and 96 h LC_{50} for orange- spotted grouper larvae

1 g KCO Loading (g/L)									
	Replicates								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
72 h LC_{50}	1.747	5.477	1.269	0.975	0.902	0.871	0.621	0.681	0.651
96 h LC_{50}	0.894	2.973	0.743	0.69	0.784	0.748	0.45	0.616	0.509
20 g KCO Loading (g/L)									
	Replicates								
	1	**2**	**3**	**4**					
72 h LC_{50}	19.5	19.6	18.4	42.3					
96 h LC_{50}	6.7	6.0	5.3	5.1					
CE-WAFs LC_{50} (g/L) Comparison									
Corexit® 9500 CE-WAF LC_{50} (g/L)									
	Replicates								
	1	**2**	**3**	**4**	**5**				
72 h LC_{50}	0.34	0.65	0.30	0.33	0.44				
96 h LC_{50}	0.17	0.41	0.19	0.11	0.18				
Corexit® 9527 CE-WAF LC_{50} (g/L)									
	Replicates								
	1	**2**	**3**	**4**					
72 h LC_{50}	0.16	0.11	0.12	0.13					
96 h LC_{50}	0.10	0.07	0.08	0.11					
Slickgone® CE-WAF LC_{50} (g/L)									
	Replicates								
	1	**2**	**3**	**4**					
72 h LC_{50}	0.4	1.3	3.4	1.2					
96 h LC_{50}	0.3	1.0	3.0	1.0					

4. 13 Discussion

Generally, fish toxicity data are highly variable because of many other factors such as maturity, species, size that govern the overall sensitivity of a test system. Different fish species and life stages have variable responses to the toxic action of dispersed and un-dispersed crude oils (NRC, 2005). The early life stages of fish tend to be the most sensitive stages after exposure to crude oil and comparisons of toxicity results among test species and their life stages, or 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). In this study, recommended methods to use WAF for toxicity testing were followed because it is the soluble fraction that enters an aquatic environment with the greatest ease and as a result can cause direct acute damage on aquatic organisms (Martinez-Jeronimo et al., 2005). Also, many fish species have been reported as demonstrating sensitive responses to such chemicals, but the relative toxic effects of these mixtures on sea bream, orange-spotted grouper, yellow-fin sea bream, and mullet life stages to our knowledge have not been investigated. Numerous methods for the preparation of WAF have been reported in the literature i.e. UNEP (1989) method uses single oil: water (1:10) loading for the preparation of WAF and subsequently serial dilution of WAF was done for the exposure. However, recently OSB (2005) debated methods for the preparation of WAF. Two distinct preparations methods for WAF of crude oil have been discussed and evaluated heavily by the scientific community engaged in research on oil toxicity (Singer et al., 2000; 2001a; Barron and Ka'aihue, 2003). The CROSERF protocols, recommend preparation of toxicity test solutions by variable loading using a series of decreasing concentrations of applied oil. Others like Barron and Ka'aihue (2003) have suggested the use of single oil: water loading rate and the preparation of test solutions using variable dilutions of the stock solution as proposed by UNEP (1989). The decision of which method to use may depend ultimately on the specific scientific question being addressed. Singer et al., (2001a) prefer for the variable loading method because they believe it is more "field relevant" since spilled oil slicks tend to be dynamic, continually changing in size, shape, and thickness. Therefore, these tests address the question: "At what oil to water loading ratio is WAF toxic?" Barron and Ka'aihue (2003) support a variable dilution system for preparing a WAF for testing dispersant that standardizes the oil: water ratio and provides a consistent chemical

concentration in a test-series for each oil-dispersant combination. Because it has not been conclusively demonstrated that either method more accurately reproduces the dilution of dispersed oil under actual spill conditions, the WAF in the present study was prepared by both methods i.e., by variable oil loading using a series of decreasing concentration of applied oil in sea water according CROSERF protocols (OSB, 2005) and by single loading followed by variable dilutions (UNEP, 1989; Barron and Ka'aihue, 2003). Other factors like mixing energies, head space, and percent vortex were kept constant by procuring the same type of aspirator bottles, same size of Teflon coated stirrer and required number of magnetic stirrers from the same company; and depending on experiments some factors were changed to examine their effects of WAF/ CE-WAF preparations.

In the present study, four fish species sea bream, orange-spotted grouper, yellow-fin sea bream and mullet were used for exposure studies. Most of the work was done on sea bream because of its ready availability; whereas orange-spotted grouper was used for comparison of the two species. Eggs and larvae of all fish species were exposed to the WAF/CE-WAF prepared by various oil loadings and subsequent dilutions at nominal concentrations which were not renewed every day (static exposure). Therefore exposure concentrations decreased as time progressed from 0 to 96 h which replicate natural scenario in the marine environment as an oil spill undergo dilution and evaporation effects. Of the fish species tested, certain life stages were affected more than another when used in toxicity tests depending upon the availability of specific stage such as embryonated egg (E), larvae hatched during exposure (LHE) and/or pre-hatched larvae (PHL). LC_{50} concentrations were determined for each day of exposure (24 to 96 h) and only 96 h LC_{50} values were used for comparison purposes among all the species. In some cases, 72 and 96 h LC_{50} values were compared to understand which one provided a better representation of the toxic effect of either KCO WAF or CE-WAFs.

In sea bream and orange-spotted grouper, most of the embryonated eggs hatched successfully at 24 h in control seawater, and the remaining eggs that were not hatched in the first 24 h of exposure hatched by 48 h; and eggs which didn't hatch beyond the 48 h period were considered dead.

Sea bream and orange-spotted grouper embryonated eggs were resistant to the toxicity of KCO WAF based on LC_{50} values and hatching was successful. Heintz (2000) have reported contrary findings to the ones in this study, in which Exxon Valdes crude

oil was extremely toxic to pink salmon (*Oncorhynchus gorbuscha*) embryos. The resistance of fish eggs was probably because of the presence of egg envelope (chorion) which serves as a protection to the fish from external chemical, physical and biological stressors in the marine environment (Yamagami et al., 1994). The chorion protection ability is mainly caused by the presence of an enzyme transglutaminase (TGase) which is responsible for hardening the egg envelope (chorion) (Ha and Iuchi, 1998). Moreover, in salmonid eggs for instance once they are ovulated, the ovarian fluid provides more protection from external disturbances until spawning stage commences. After fertilization, vitelline membrane, chorion and the the enveloping layer are the main structures which protects the embryo from poor water quality. The vitelline membrane is one of the least permeable membranes, while the chorion is semi-permeable and it provides certain defense mechanism against xenobiotic chemical intoxication (Finn, 2007).

Larvae hatched during exposure survived in control seawater up to 96 h duration of the test. In preliminary experiments, survival percentage of laboratory hatched larvae was low which improved with improvement in exposure conditions. In later experiments, around 100% hatching success was achieved and the resultant larvae survived well up to 96-h exposure period. A variation in egg hatching and larval survival in controls reflected the quality of eggs at the commencement of the experiment. After standardization of assay conditions in controls, the exposure to KCO WAF and CE-WAF was conducted and compared with the observations in control. Larvae hatched during exposure to KCO WAF (1 g KCO/L seawater) seemed to be vulnerable to its toxicity as was reflected through the overall health status of each larva which didn't exhibit successful survival at 96 h of exposure compared to healthy larvae in the control.

It has been noticed that preparation method influenced the composition of WAF and/CE-WAF and in turn its toxicity. TPH concentrations in KCO WAF or CE-WAF prepared by variable oil loadings were not linear to the amount of oil loaded possibly because of the different solubility of individual crude oil components due to different oil: water ratios. On the other hand, when WAF prepared using single oil loading and successive dilutions were made, linearity in TPH concentrations was observed in various dilutions. Since preparation method can influence the toxicity of either WAF or CE-WAF solutions, it is largely debated in scientific literature that which method might clearly demonstrate the true toxic effect of WAF solution.

LC_{50} values indicated that the toxic effect of WAF was more on larval survival than on egg hatching and the response of orange-spotted grouper was different from sea bream where sea bream hatched larvae showed maximum sensitivity to KCO WAF.

When WAF prepared at different oil loadings (1-80g KCO/l seawater) with subsequent serial dilutions of each loading separately, an interesting pattern emerged. The most toxic WAFs on sea bream PHL were found to be those prepared at lowest oil loading i.e., 1 g KCO/L seawater which was in agreement to what Barron et al., (2002) observed that WAF solution was acutely lethal when prepared with only 0.01 to 0.1 g oil/L seawater applied oil loading, except in the Barron case; his oil loading was even lower compared to the one used in this study. WAF prepared with increasing oil loadings (≥ 1 g KCO/L seawater) with serial dilutions was not found to exert increasing toxic effects indicating that saturation of water soluble compounds was achieved at 1 g KCO/L seawater oil loadings, and further increase in oil content could not increase partitioning of water soluble compounds in the aqueous medium. This was substantiated by studies on the chemical characterization of WAF reported (Chapter Three). Other workers also encountered this difficulty in the preparation of WAF fraction that oil to dissolving medium ratios did not increased the TPH content in WAF (Navas et al., 2006; Gonzales-Doncel et al., 2008). Therefore, in determining the toxicity of oil; it is important to consider the oil-water ratio. That means in a spill scenario, the spread of oil over the water will be an important consideration in determining the risk to water column organisms. In our experiments, it was observed that 1 g KCO/L seawater loading was most suitable for WAF preparation because upon dispersant treatment, the KCO CE-WAF was turbid at higher concentrations of oil loadings making it difficult to use in toxicity assay, especially when attempting to count either egg or larvae as they are not visible under the turbid solution. More so, comparing the LC_{50} of WAF prepared at 1 g KCO/L seawater with WAF prepared at higher loading (20 g KCO/L seawater) for instance, it showed that LC_{50} values were higher in the higher oil loadings indicating less toxicity. A possible explanation is that, partitioning of KCO in the aqueous phase was not increased possibly because of fixed surface area of underlying seawater in the WAF preparation bottles and the slow stirring speed used.

When the toxicities of variable oil loadings of KCO WAF were compared against sea bream, orange-spotted grouper, yellow-fins sea bream, and mullet pre-hatched larvae, it revealed that 1 g KCO/L seawater WAF loading was more toxic to sea bream pre-hatched larvae than to orange-spotted grouper; whereas 20 g KCO/L

seawater WAF loading exerted almost similar toxicity effects on, sea bream and orange-spotted grouper followed by yellow-fin sea bream which was the least sensitive against that loading. Further tests with higher KCO WAF loadings such as: 1, 10, 20, 40 and 80 g KCO/L seawater on sea bream pre-hatched larvae were conducted in anticipation that some compounds which have not been estimated in routine chemical analysis might have been partitioned in WAF and may exert toxic effects. It revealed that WAF of 1 g KCO/L seawater oil loading was the most toxic to sea bream pre-hatched larvae and increasing KCO loadings didn't enhance its toxicity. Others like Al-Yaqoob et al., (1996) observed differing responses of other fish species to KCO toxicity in which it induced more toxic effect on inland silverside (*Menidia beryllina*) fish than what was observed for orange-spotted grouper. Crimson-spotted rainbow hatched larval stage (*Melanotaenia fluviatilis*) responded differently to WAF toxicity in which it was highly toxic to this stage (Pollino and Holdway, 2002).

When LC_{50} values were calculated on the basis of oil loadings, the toxicity seemed to decrease with increasing oil loaded on the water, as the LC_{50} values increased because the oil loaded for WAF preparations was high. This ambiguity was resolved when the LC_{50} values were equated in terms of TPH in the WAF and the values obtained were close for all WAF preparations which were made at different oil loadings. This further substantiated our assumption that after saturation at about 1 g KCO/L seawater in our preparation vessel; considerable increase in toxic compounds partitioned in water phase was not observed by increasing the oil loading and that explain why LC_{50} values remained more or less the same in different exposure of prepared WAF in terms of TPH values. And these findings are in accordance with other literature as mentioned previously where difficulty was encountered when increasing oil loading leading to minimum increase in TPH concentrations in WAF solutions (Navas et al., 2006; Gonzales-Doncel et al., 2008). This study suggests that in determining the toxicity of oil, it is important to consider oil-water ratio; meaning that in the oil spill site, the spread of oil over the water will be an essential consideration in determining the risk to water column organisms such as fish species. We continued exposure assays with WAF of higher oil loading in anticipation that some compounds which have not been estimated in routine chemical analysis might have been partitioned in WAF and may exert toxic effects other than mortality on biota.

In another set of experiments, exposure of sea bream to KCO WAF prepared at 1 to 20 g KCO /L seawater loadings revealed that the percentage of egg hatching at 24 h

was not proportional with the various oil loadings. Thus, producing an irregular pattern; but it improved by 48 h and was still not consistent with the increasing oil loadings. Meanwhile, the survival success of the sea bream larvae which hatched during exposure to WAF of variable oil loadings varied and exhibited a concentration dependent effect; but the effect was not linear with the increasing oil loadings too. KCO WAF was more toxic to sea bream larvae than to mullet exposed to the same oil loading (1 to 20 g KCO /L seawater), but it was less toxic compared to the WAF's of Alaska North Slope and Weathered Venezuelan medium crude oils against inland silverside (*Menidia beryllina*) and red drum (*Sciaenops acellatus*) (Rhoton et al., 2001; Wetzel and Van Fleet, 2001). KCO WAF, appeared more toxic than what was found for the WAF of Bass Strait crude oil using Australian bass (*Macquaria novemaculeata*) (Gulec and Holdway, 2000).

Sea bream life stages responded differently to KCO WAF, and it was more toxic to Pre-Hatched Larvae (PHL), followed by Larvae Hatched during Exposure of eggs (LHE), and finally Embryonated egg stage (E) which was the most resistant stage to the toxic effect of KCO WAF. In many studies, the discrepancy between sensitivity to hydrocarbons and development stage are discussed and most of the observations documented that those larvae and young fry are more sensitive to the water-soluble fraction (WSF) concentrations than eggs did (Kunhold, 1970; Struhsaker et al., 1974; Moles et al., 1979). Several other studies suggested that, in early development; damage to a few precursor cells will result in more broad damage to the overall health of the fish exposed (Rosenthal and Alderdice, 1976; Longwell, 1977). Similarly, Carls and Rice (1988) have observed that walleye pollock (*Theragra chalcogramma*) larvae were significantly more sensitive to the WSF of Cook Inlet crude oil than developing embryos, since eggs bioaccumulated much less hydrocarbon than the larval stage did and larvae were rapidly killed by the WSF solution; but exposed embryos didn't die until after hatching. In addition, Linden (1978) observed that fertilization of Baltic herring (*Clupea harengus*) was generally not affected by exposure to WSF. It has been observed too that fish early life stages tend to be the most sensitive stages after exposure to crude oil (Shales, 1989; Norcross et al., 1996). Polloino and Holdway (2002) have observed developmental abnormalities which affected survival prior and after hatching in a sense that hatchability and the incidence of larval deformity were decreased at a TPH concentration of $\geq$ 0.5 mg/L seawater ($\geq$0.0005 g/L). In addition, exposure of rainbow fish to petroleum hydrocarbons resulted in abnormal jaw development post hatching as a consequence of initial toxic exposure to crude oil.

We have also prepared WAF of the same volume of dispersant used for later CE-WAF preparations and it was tested for its toxicity. When WAF of 0.0049 to 0.078 g Corexist® 9527/L seawater dispersant loading was investigated to initially understand the toxicity of that dispersant, it revealed that the dispersant WAF exerted toxicity in a similar manner to all of the three sea bream life stages which were: embryonated eggs (E), larvae hatched during exposure (LHE), and pre-hatched larvae (PHL). Others like Slade (1982) have observed that Corexist® 9527 was toxic to embryo-larval spot fish (*Leiostomus xanthurus*) and Briceno et al., (1992) have found that Corexist® 9527 exerted some effect on inland silverside (*Menidia beryllina*) larvae.

For toxicological assays, CE-WAF prepared by variable oil loadings and CE-WAF prepared by single oil loading with subsequent serial dilutions of Corexist® 9527 dispersant were used. It interestingly exhibited some differences in the toxic responses of either egg or larval stages. Test conducted with CE-WAF prepared at variable oil loadings and oil dispersants Corexit® 9527 at 10:1 (oil: dispersant) ratio, showed that higher WAF oil loadings exerted toxic effects on sea bream egg hatching and none of the egg hatched. In general, CE-WAF prepared by variable loading method appeared to be less toxic than what was observed using serial dilution preparation method and variable loading was less toxic to the egg stage (E), followed by larvae hatched during exposure (LHE), and then pre-hatched larvae (PHL). The TPH concentrations for CE-WAF prepared by variable oil loadings were not linear to the oil loadings. Whereas, CE-WAF prepared using single oil loading and subsequent dilutions indicated linearity in TPH concentration. A better TPH concentration linearity was achieved in serial dilution method, and its toxicity was higher than what was observed for the variable oil loading method. Considering linearity with TPH values in CE-WAF dilution series, this method was preferred in various comparison studies and concentration-response relationship was observed.

The effect of CE-WAF mixtures on fish species examined were variable, species and life stage dependant. The change in the order of toxicity of CE-WAF mixtures may be related to the different degradation rates and/ or degradation products of the dispersant, indicating that toxicity data vary for different oil dispersants and different crude oil types (Pollino and Holdaway, 2002). Barron et al., (2004) indicated that, the toxicity of WAF and CE-WAF solutions were similar in exposed fish egg and larvae, while other studies have demonstrated mixed responses and 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).

When all the three CE-WAFs prepared using three oil dispersants were tested individually, their toxicities against sea bream and orange-spotted grouper were of different degrees to the two fish species; as in some cases the 96 h LC_{50} values decreased compared to KCO WAF alone. Singer et al., (2000) demonstrated that the primary function of oil spill dispersant was to increase the entry of oil into water column thus modifying the exposure medium and increasing its toxicity. Dispersion of crude oil with oil dispersant (CE-WAF) have increased its toxicity in comparison to the toxicity 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). Results obtained in this study were variable for three CE-WAF solutions and principally, when living organisms are exposed to two or more chemicals simultaneously; the specific interaction between the constituents may result in an enhancement the ultimate effect of the toxic chemicals (Cluevers, 2003; Otitolujo, 2003; 2005; Samuel et al., 2008). Pollino and Holdway (2002) also observed an increase in larval mortality with time in a 96 h exposure period to crude oil WAF and CE-WAF. However, in our study CE-WAF caused a sharp decrease in 24 h LC_{50} values compared to KCO WAF; indicating quick enhancement of toxicity of KCO by treatment with dispersants for orange-spotted grouper for instance. At 48 h, the LC_{50} values of WAF and CE-WAF were comparable, and with time the severity in toxicity increased with both WAF and CE-WAF. Chemical dispersants possess the ability to enhance oil spill dispersion by forming water-accommodated micelles with oil droplets; that facilitate the uptake and accumulation of hydrocarbons in the organism. The mechanism by which dispersants alter hydrocarbon bioaccumulation process is not well understood (Mielbrecht et al., 2005). The increase in hydrocarbon concentrations in CE-WAF may be responsible for the enhancement of toxicity. However, our data suggest that, in oil spill scenario if any sensitive species is present in the spill area, the use of dispersant is not advisable since it quickly increases the toxicity of chemically enhanced oil whereas oil alone takes around 48 h to exert similar effects and that period may provide window to save the sensitive species.

Others like, George-Ares et al., (2000) indicated that Corexit® 9500 and 9527 dispersants are of a low to moderate toxicities when tested on most aquatic species, and many factors may contribute to the test results variability such as species, exposure duration and etc. this finding is in agreement to what was obtained for sea bream in a

way that KCO WAF of similar toxicity to Corexit® 9527 CE-WAF and Corexit® 9500 CE-WAF was less toxic.

Findings in our study in relation to the increased sensitivity of fish larval stages more than the embryonic stages are in agreement with literature as was observed, and that Corexit® 9500 and 9527 and Slickgone® CE-WAF's were generally more toxic in different magnitudes to orange-spotted grouper pre-hatched larvae (PHL), followed by larvae hatched during exposure (LHE), then embryonated egg stage (E). Those finding is in agreement with what Paine et al., (1992) observed, that hydrocarbons, being lipophilic in nature, accumulated primarily in the yolk of embryos, and as a result, their effects may not appear until the yolk is utilized by older embryos and larvae. In this study, larvae hatched during exposure exhibited more sensitivity to WAF than egg stage because of hydrocarbon accumulation which made it more susceptible to toxicant; same to what Fucik et al., (1995) have demonstrated that tests conducted with inland silverside fish (*Menidia beryllina*) under static exposure system to two Gulf of Mexico oils, dispersed oil mixtures, and Corexit® 9527 dispersant, showed that larval stages were more sensitive to the toxic mixtures than embryos with dispersant being the most toxic, followed by dispersed oil, then WAF of crude oil which was the least toxic. Hatching rates were reduced in a 25 and 50% WAF, and the reduction was attributed to the effects of higher exposure concentrations.

In general, the overall toxicity pattern of the CE-WAF solutions compared to that of KCO against sea bream pre-hatched larvae was as follows: KCO WAF was the similarly toxic to Corexit® 9527 CE-WAF, followed by Corexit® 9500 CE-WAF, then Slickgone® CE-WAF.

For orange-spotted grouper, the toxicity pattern was as follows: Corexit® 9527 CE-WAF was the most toxic chemical, followed by Corexit® 9500 CE-WAF, then Slickgone® CE-WAF, then KCO WAF, although Corexit® 9527 CE-WAF was of similar toxicity to that of Corexit® 9500 CE-WAF. The pattern obtained with orange-spotted grouper was in agreement with Lönning and Hagström (1976) in which they observed that the combination of oil and Corexist® dispersants proved to be more toxic to embryonic and the larval stages than oil by itself. Cohen and Nugegoda (2000) findings have indicated that after exposure of fish to Bass Straight crude oil treated with Corexit® 9527 dispersant, the CE-WAF solution was more toxic than crude oil WAF which was similar to our findings. Clark et al., (2001) on the other hand, observed that KCO dispersed with Corexit® 9527 was more toxic to turbot and inland silverside

embryos and larvae than what was found in this study. When Pollino and Holdway (2002) used crimson-spotted rainbow fish, they found that Corexit® 9527 CE-WAF was more toxic than both Corexit® 9500 CE-WAF and KCO WAF. Jung et al., (2009) have also confirmed that the addition of dispersants to crude oil will enhance the concentration of hydrocarbons available to ovoviviparous rockfish (*Sebastes schlegeli*) as the concentrations of cytochrome P450- 1A and EROD activity were increased in the fish after exposure to crude oil WAF after dispersing the crude oil with Corexit® 9500 dispersant.

Although LC_{50} of CE-WAFs with all the three dispersants were above the ecotoxicological criteria, but sensitivity of early life stages of fish demands a care in the application of dispersant close to fish breeding areas and nurseries. The findings in this study explain the main concept that joint-toxicity occurs when two chemicals (crude oil + dispersant) exert their effects simultaneously (NRC, 1989).

When LC_{50} values for sea bream were calculated on the basis of oil loading, values were of comparable toxicity for Corexit® 9527 CE-WAF and KCO WAF with KCO WAF being slightly toxic, but it were less toxic for Corexit® 9500 then Slickgone® CE-WAF. On the other hand, when CE-WAF values were equated using TPH values (analyzed by FT-IR method); KCO WAF was the most toxic and Corexit® 9527 and Slickgone® CE-WAF were of comparable toxicity against sea bream except Corexit® 9500 was less toxic.

CE-WAFs were more toxic to orange-spotted grouper as compared to KCO WAF, but when CE-WAF values were equated using TPH values (analyzed by FT-IR method) KCO WAF, Corexit® 9527 and Slickgone® CE-WAF were of comparable toxicity except Corexit® 9500 was less toxic and all were of close values.

Although, TPH concentration (FT-IR) were the highest for Corexit® 9500 CE-WAF among other CE-WAFs and WAF, but its toxicity when equated in terms of TPH was the lowest against sea bream, this indicates that TPH is not an accurate method of chemically quantifying WAF and/ or CE-WAF and to date there is no accurate method in literature as mentioned earlier. It is more appropriate and indicative of toxicity nature to express LC_{50} results based on oil loadings to achieve a better understanding of test chemical behavior.

Since fish larval mortality was observed in an aqueous medium in this study as in many other studies, toxicity can be largely attributed to dissolved PAHs in WAF and/CE-WAF solutions. PAHs dissolved from oil medium were toxic to zebra fish

(*Danio rerio*) and physical contact of embryo with oil droplets did not contribute to embryotoxicity. This is supported by the findings which indicate that oil particles in mechanically-dispersed Alaska North Slope crude oil drift past the embryonated egg chorion membrane without adhering to its surface. Because, zebra fish and pink salmon both have chorion membranes with pore sizes small enough to inhibit the entrance of most if not all oil particles (Stehr and Hawkes, 1979; Darie et al., 2004; Monné et al., 2006; Cheng et al., 2007; Carls et al., 2008). Other factors like water quality parameters such as salinity and water temperature can also influence embryonic development, hatching, feeding and survival of fish larvae (Jennings and Pawson, 1991; Watanbe et al., 1995; Ibrahim et al., 2010).

Heintz et al., (1999) further supported those findings and indicated that, PAH accumulation and embryo mortality in pink salmon in direct contact with gravel covered with oil; were not significantly variable from accumulation and mortality in embryos induced by exposure to dissolved PAHs in effluent water. Therefore, test chemicals dissolved in solution are more bioavailable than chemicals of solid or adsorbed nature to solid objects (Golding et al., 2007).

Others have demonstrated contrary findings, in which higher toxicity of WAF prepared at less than 1 g KCO/L loading maybe was because of non-dissolved dispersed oil droplets. Those findings are in agreement with data reported by Girling et al., (1992) in which they observed that petroleum hydrocarbon products which didn't undergo complete dissolution in the aqueous phase, but were dispersed; adversely affected marine organisms because of the joint-effect of toxicity and physical fouling.

Exposure of sea bream larvae to KCO WAF and CE-WAFs resulted in variable LC_{50} concentrations for each exposure time (24, 48, 72 and 96 h). However, in general it was observed in this study that 72 h LC_{50} values obtained from different experimental replicates were in general more consistent with each other. This was probably because of the survival of exposed larvae between 72 and 96 h was inconsistent to exposure concentrations and a sharp decline in 96 h LC_{50} concentrations was observed. Therefore, it is considered that the for sea bream larvae, 72 h LC_{50} are a better representatives of KCO WAF and CE-WAF toxicity. Whereas, LC_{50} concentrations obtained for 96 h showed an irregular pattern between exposure replicates. This ambiguity was not observed for exposed orange-spotted grouper larvae, where generally in both 72 and 96 h LC_{50} values were consistent between exposure replicates. This difference in response

to WAF and CE-WAF of KCO exposure, suggests differences in species sensitivities to exposure stress.

Exposure to petroleum hydrocarbons can lead to malformations in embryo-larval fish stages because of enzyme inhibition by pollutants. Deformity types can encompass the following: jaw abnormalities, eye deformations, disturbed vertebral column, and abnormal behaviors like impaired catching behaviors, slower growth rate, impaired swimming patterns, reduced length, and increased susceptibility (von Westernhagen, 1988). There is no visual evidence recorded in literature of sea bream larvae developmental malformations associated with exposure to water-accommodated fractions of Kuwait crude oil. Therefore in this study, KCO WAF prepared with a single loading and serial dilution caused developmental malformations in all of exposure concentrations with lordosis, kyphosis and scoliosis as the common developmental abnormality types encountered with additional other deformity types that were observed occasionally. WAF prepared at single KCO loading demonstrated a linear response in deformity percentages of larvae expose to dilution series. This further substantiated that in our WAF preparation condition, 1 g KCO/L was the minimum required oil loading to produce stable WAF devoid of micro-oil droplets. Deformity types encountered in this study are in accordance with the ones documented in literature (Jezierska et al., 2000). Pollino and Holdway (2002) observed that exposure of fish to petroleum hydrocarbons resulted in abnormal jaw development after hatching. Spotted halibut (*Verasper variegatus*) exposed to heavy crude oil which flows from oil tankers or coastal industry demonstrated delayed development, abnormal development of head morphology, smaller craniofacial and eye structures, and abnormal neural development. Heavy crude oil highly affect embryo differentiation and cell proliferation (Murakami et al., 2008). Incardona et al. (2009) found that exposure of Pacifc herring (*Clupea pallasi*) to effluents of oil gravel column developed edeman and cardiac arrhythmia because of the presecnce of tricyclic PAH. Heart shape changes and reduction in swimming performance of adult zebrafish after exposure at early embryonic stages to low concentrations of crude oil resulted in subleathal and delayed effects (Hicken et al. 2011).

4. 14 Conclusion

The present study aimed at determining the toxicity of water accommodated fraction (WAF) and chemically enhanced water-accommodated fraction (CE-WAF) of Kuwait crude oil (KCO) exposure on fish egg hatching and the survival of larvae, to gather evidence of the lethal concentrations that causes mortality in fish egg and larvae and to gain insight into the causative components in KCO WAF/CE-WAF responsible for causing harmful effects on the early life stages of fish.

Successful hatching of sea bream and orange-spotted grouper embryonated eggs after exposure to KCO WAF and CE-WAF was observed, but larvae hatched during exposure and pre-hatched larvae were more sensitive to WAF/CE-WAF toxicity than embryonated eggs.

The most toxic WAF was found to be the one prepared at lowest oil loading. WAF prepared with increasing oil loading was not found to exert increasing toxic effects, indicating that saturation of water soluble compounds was achieved at 1 g KCO/L seawater loadings, and further increase in oil loading could not considerably increase partitioning of water soluble compounds in the aqueous medium. An increase of chemicals partitioned in aqueous phase was observed after using KCO WAF prepared at 1 g oil/L seawater loading which might have caused its toxicity more than was observed for higher oil loadings.

Toxic responses of fish larvae after exposure to KCO WAF and CE-WAF using three individual dispersants were species and test chemical dependent. KCO WAF was as similarly toxic as Corexit® 9527 CE-WAF against sea bream, but Corexit® 9500 and Slickgone® CE-WAF were of lower toxicity, so dispersant didn't seem to enhance KCO toxicity. On the other hand, CE-WAFs were more toxic on orange-spotted grouper as compared to KCO WAF, thus; the addition of dispersants has increased KCO toxicity.

When LC_{50} values of KCO WAF using sea bream larvae were calculated on the basis of oil loadings, their toxicities seems to decrease with increasing oil loaded on the water as the LC_{50} values increased because the oil loaded for WAF preparations was high, but when the LC_{50} values were equated in terms of TPH in the WAF the values were very close at all the WAF preparations made at different oil loadings. Thus, indicating that increasing oil loadings have not increased the toxicity of KCO WAF solution.

Exposure to KCO WAF have demonstrated sublethal effects in sea bream fish larvae in all exposure concentrations and various types of developmental deformities specifically in body curvature, delayed growth and abnormal jaw were recorded. Petroleum hydrocarbon concentration in the Kuwait marine area normally range from 0.00105 to 0.0266 mg/l seawater with average 0.00236 mg/L seawater (EPA Kuwait, 2008) is much lower than LC_{50} values obtained sea bream and orange-spotted grouper larvae. Therefore, this study demonstrated that the current contamination of petroleum hydrocarbon in Kuwait's marine area does not pose any acute hazard to fish egg hatching or the survival of hatched larvae. However, caution is required, since occasionally EPA reported episodic contamination that may cause mortalities in developing larvae. In addition, in chronic exposure a more serious effect may be anticipated on larval growth due to sub-lethal effects of dissolved hydrocarbons. Since Kuwait crude oil is used for oil spill dispersants evaluation and approval, the generated data concerning Kuwait crude oil will further assist regulatory authorities in understanding the behavior of crude oil in the case of oil spill scenario (www.defra.gov.uk, 2007).

In order to protect marine fish species local to Kuwait territorial waters against oil contamination, the LC_{50} concentrations generated in this study should be considered as an indicator for marine pollution, and any concentration exceeding those values, may eventually cause an adverse effect.

Chapter Five

GENERAL DISCUSSION

5. 1 General Discussion

The toxic effects of crude oil, dispersed oil and chemical dispersants especially on marine fish have been discussed thoroughly in literature (Smith et al., 2006), and chemical dispersants have gained a broad approval as a potentially effective and beneficial technique in the oil spill response strategy. However, the toxic effects of those chemicals on marine fish local to the Arabian Gulf region, specifically to Kuwait are not known. Toxicity data pertaining to fish are highly variable because of many other factors such as maturity, species, and size that govern the overall sensitivity of a test system and different fish species with different life stages have variable responses to the toxic actions of dispersed and un-dispersed crude oils (NRC, 2005). Therefore, in this study; toxicity of water-accommodated fraction of Kuwait crude oil (WAF) and chemically enhanced water-accommodated fraction (CE-WAF) of Kuwait crude oil plus three oil dispersants were investigated against native marine fish species to understand the nature of chemical preparations in environmental conditions similar to Kuwait marine environment.

The study was comprised of two parts which were chemical characterization of KCO WAF and CE-WAF solutions, and their toxicity against marine fish early life stages. Characterization experiments were conducted to understand the nature of WAF and CE-WAF in aqueous medium, and the effect of the variable test settings on changes in volatile component (BTEX) and TPH concentrations partitioned in aqueous phase from crude oil. Several outcomes were achieved from these experiments in which BTEX and TPH concentrations were increased with the increasing oil to water loadings, but the increase was not proportional to the oil loadings. The effect of two mixing speeds and two mixing times resulted in a variable BTEX and TPH concentrations attributable to different preparation settings, and a low mixing speed was found adequate for the preparation of KCO WAF because it contained a reproducible BTEX and TPH concentrations. The effect of single oil loading and multiple mixing times on KCO WAF preparation indicated that BTEX and TPH compounds demonstrated a gradual increase until saturation, and a 24 h period was found to be the most appropriate mixing time to generate a reproducible WAF solution. When KCO was treated with

three dispersants separately and CE-WAF solutions were prepared under identical conditions, BTEX concentrations were variable in all preparations, and TPH levels in CE-WAF preparations were higher than that of KCO WAF alone. Further tests were conducted by initiating seawater stirring in the mixing vessel and delivering the oil/dispersants in the vortex (DNIV) and it was not found to be advantageous over the other method which suggests layering of oil/dispersant on the surface of the water and then starting seawater stirring (DNIV) (Singer et al., 2000). Thus, the optimum conditions followed after standardizing the preparation of stable WAF/CE-WAF solutions were the following: 1 g KCO/L seawater, 1:10 oil: dispersant ratio, delivery of oil/dispersant not in the vortex, 24 h mixing duration, and low mixing speed. The greatest challenges in this study comes from the need to standardize KCO WAF preparation procedure since there is no common universal preparation methodology for WAF preparation in laboratory to replicate natural oil spill scenarios making findings from different authors rather difficult to compare and these limitations were encountered by others (Singer et al., 2000). That is why we have attempted to standardize the conditions for Kuwait crude oil (export quality) preparation in accordance with recent attempts of other scientists attempting to standardize WAF preparation for instance by high energy mixing using sonication (Elordui-Zapatarietxe et al., 2008).

In general, dispersants noticeably increased the aqueous concentrations of PAHs, and KCO WAF concentration was close to that of Corexit® 9527 CE-WAF, but Slickgone® CE-WAF had the lowest PAH concentration. Aliphatic compounds had higher concentrations of n-alkanes which were detected in CE-WAF but not in WAF.

TPH determined by FT-IR and Spectrofluorophotometry revealed that, in general; the two methods were in agreement with each other in which KCO CE-WAFs had higher TPH concentrations than KCO WAF which indicate that the additions of dispersants have increased the dissolution of crude oil in the aqueous phase. The three dispersants tested have different potentials of dispersing crude oil and the dispersion process have increased the partitioning of certain petroleum hydrocarbons into the aqueous phase.

The chemical compositions of WAF and CE-WAF solutions was influenced by preparation methods used leading to variable toxicity results. When WAF was prepared using single KCO loading and successive serial dilutions made, a better linearity in TPH concentrations was observed compared to TPH concentrations obtained from

preparation using variable oil loadings. Therefore, the serial dilution of WAF prepared by single oil loading was used for toxicity bioassays against selected fish species. Before toxicity testing, the stability of serially diluted WAF and CE-WAF solutions in 96-h bioassay containers was examined and the decrease in TPH percentage during test period was determined. In CE-WAF, the decrease with time was more than what was observed for KCO WAF over a 96-h test period. However, for LC_{50} calculation, nominal concentrations were used.

It is to be emphasized that crude oil is a complex mixture of organic compounds and a variety of factors can influence the partitioning of compounds into the aqueous phase. In our study, attempts have been made to keep constant as many factors as possible during WAF/CE-WAF preparation like salinity and temperature to get a stable solution to be used for experimental exposure. However, variations in salinity and temperature not only can influence the partitioning of compounds in the aqueous phase, but also; it can change the toxicity of prepared WAF/CE-WAF. It has been reported that aromatic hydrocarbons solubility can be increased as water temperature increases and salinity decreases (May and Miller, 1981; Schwarzenbach et al., 2003, Elordui-Zapatarietxe et al., 2008). Therefore, further studies on the effect of salinity and temperature during WAF preparation on the solubility of Kuwait crude oil and its relative toxicity on fish larvae may provide interesting observations. In addition, It is important to understand the fate of petroleum hydrocarbons dissolved in the water phase and it's accumulation in bottom marine sediment (sediment toxicity), because sediments frequently contain higher concentration of pollutants than the surrounding water medium as liphophilic organics tend to adsorb on particulate matter that may suspend in the water column (Karacik et al., 2009). Others have preferred using different exposure systems for the detection of toxic effects on fish and although, the effects of open test chambers (acute-static) exposure systems using high oil/dispersant dosage was investigated in this study. It is necessary to examine the effect of long-term chronic exposure of low oil/dispersant dosage on fish by using closed flow-through test systems on fish structural and developmental abnormalities and alterations in fish behavior and swimming performance. Outcomes of those experiments can shed light on which test system might have the potential to better maintain the concentration of chemical compounds in aqueous medium. Selection of which exposure system to be used in toxicity testing depends on the objective of the experiment as which system can play a significant role in inducing the most toxic effect on the exposed fish, since flow-through

system can be sometimes preferred by other scientists specifically if they are dealing with chemicals that are unstable, volatile and with high oxygen demand (Becker and Crass, 1992; Lammer et al., 2009).

Fish have been used as a toxicity model since they tend to accumulate and metabolize petroleum hydrocarbons. They are also considered as a suitable bioindicators for environmental pollution in aquatic systems because hydrocarbons are accumulated more in exposed organisms rather than in the surrounding environment (Gravato and Santos, 2002; Anyakora et al., 2005). In this study, the toxicity was investigated by conducting a 96-h acute toxicity test against different early-life stages of selected marine fish species native to Kuwait and fish lethality/survival were examined as the end point. However, there is a scope to conduct further research on the sublethal effects of WAF/CE-WAF since oil dispersants and PAHs have the capacity to transform into endocrine disruptors and become cytotoxic which could have a severe impact on marine organisms and affect human health (Evanson and Van Der Kraak, 2001; Kennedy and Farrell, 2006; Judson et al., 2010). In addition, short term exposure can enhance respiratory burst activity (RBA) in Pacific herring (*Clupea pallasi*), but sub-chronic exposure can reduce RBA (Kennedy and Farrell, 2008); therefore comparison of acute hydrocarbons exposures with sub-chronic ones can further assist in the determination of subsequent sublethal effects.

KCO WAF prepared with increasing oil loadings (> 1 g KCO/L seawater) with serial dilutions was not found to exert increasing toxic effects on sea bream pre-hatched larvae indicating saturation of water soluble compounds at this concentration. When oil loading was increased (> 1 g KCO/L seawater), minimum increase in TPH concentrations was achieved in WAF solutions, therefore toxicity of higher loadings was not enhanced as similar situations were encountered in other reported studies (Navas et al., 2006; Gonzales-Doncel et al., 2008). It was revealed that the most toxic WAF solution was found to be the one prepared at i.e., 1 g KCO/L seawater from a series of increasing oil loadings. Therefore, this loading was selected for the preparation of WAF/CE-WAF solutions and toxicity determination.

The present study indicated that exposure to WAF and CE-WAF caused no adverse effects on the successful hatching of sea bream and orange-spotted grouper embryonated eggs. Whereas, pre-hatched larvae responded to exposure and sea bream larvae were more sensitive to KCO toxicity than orange-spotted grouper. It is well documented that early life stages of fish are more susceptible to crude oil toxicity than

the adult stages, and the reason which makes early life stages of fish more sensitive than adult stages to chemical contaminants is mainly because of their undeveloped organs and large surface body area which eventually contribute to higher accumulation of petroleum hydrocarbons (Stephens et al., 1997). Meanwhile, since fish adults life stages can bioccumulate petroleum hydrocarbons inheriting in metabolic functions, examination of other life stages of fish life such as fingerlings and juveniles upon exposure to crude oil can provide a new research challenge to be compared to early-life stages.

Compared to WAF of KCO alone, CE-WAF in general exerted either equal toxicity or more toxicity on pre-hatched fish larvae depending upon the species and the dispersant used for the preparation of CE-WAF. Sea bream pre-hatched larvae showed maximum sensitivity to KCO WAF ≥ Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF, while orange-spotted grouper pre-hatched larvae were the most sensitive to Corexit® 9527 CE-WAF > Corexit® 9500 CE-WAF > Slickgone® CE-WAF > KCO WAF. Lin et al. (2009) also observed that addition of Corexit® 9500 to Prudhoe Bay crude oil didn't increase its toxicity against chinook salmon (*Onchorhyncus tshawytscha*). The difference between the toxicity of WAF and CE-WAF solutions can largely be explained by the fact that WAF alone carry less hydrocarbons into test solution than CE-WAF mixtures do, and the ways by which toxic constituents of chemicals is transferred from oil to aqueous mediums can therefore; principally control the uptake, exposure and toxic effects of those chemicals in the exposure medium (Schein et al., 2009).

Although LC_{50} of CE-WAFs with all the three dispersants were above the ecotoxicological criteria, but sensitivity of early life stages of fish demands a care in the application of dispersants close to fish breeding areas and nurseries. Reported petroleum hydrocarbon concentrations in Kuwait marine area are much lower than LC_{50} values for sea bream and orange-spotted grouper larvae obtained in this study, suggesting no immediate threat. However, caution should be taken in the application of dispersants since it is not advisable to use them near fish breeding grounds because they can increase the dissolution of oil droplets in the aqueous phase; therefore increasing its toxicity against marine organisms (EPA Kuwait, 2008).

Exposure of sea bream larvae to KCO WAF in this study resulted in various types of developmental abnormalities specifically in spinal curvature. Further investigation of other types of larval deformities upon the acute exposure to crude oil is

necessary because it was documented that oil can lead to a wide array of developmental abnormalities like bradycardyia and somite reduction (Shen et al., 2010). Long-term exposure of fish to crude oil is interesting to explore as it might provide information on growth reduction in fish and genetic damage to future fish brood, because it was demonstrated that exposure of pink salmon (*Oncorhynchus gorbuscha*) to dissolved polynuclear aromatic hydrocarbons resulted in delayed development which assisted in the continuation in the susceptibility of mechanical damage (Carls and Thadinga, 2010).

Considering the complexity of crude oil, one study can't have the potential to answer all questions regarding the toxicity of oil and dispersed oil toxicity. Since different fractions of the spilled oil exerts various immediate acute and sub-acute, further research is required to explore all the missing gaps in this research. Decisions regarding the application of dispersants in the case of an oil spill incident should depend on cumulative knowledge of crude oil and dispersed oil toxicity to minimize acute and chronic adverse impacts on marine fish species in the region.

In this study, we have developed protocols for oil and dispersed oil toxicity testing and these new protocols can be used for the screening of new chemical dispersants to be used in Kuwait's Territorial Waters to provide safety data for regulatory authorities. Findings from this study serve as building blocks for aquatic ecotoxicology in Kuwait and the Arabian Gulf region, and it opens new windows for future research studies in order to understand the effects of the following: other types of oil dispersants approved to be used in the ROPME sea area, Kuwait crude oil dispersed by those dispersants, effect of WAF and CE-WAFs on other fish life stages, and the toxicity effects on other commercially important marine fish species in the Arabian Gulf. The outcomes of this study can assists in developing new dispersants with minimum side effects on marine organisms based on the lethal and sub-lethal effects of the prepared WAF/CE-WAF solutions.

Appendices

Appendix A: Material, Safety and Data Sheets for Oil Dispersants

Table A-1: Corexit® 9500 Material, Safety and Data Sheet

(800) 424-9300 (24 Hours) CHEMTREC

1. Chemical Product and Company Identification

Product Name: **COREXIT® 9500**

Application: Oil Spill Dispersant

Company Identification: Nalco Energy Services, L.P., P.O. Box 87, Sugar Land, Texas, 77487-0087

Emergency Telephone Numbers: (800) 424-9300 (24 Hours) CHEMTREC

NFPA 704M/HMIS RATING

Health : 1 / 1 Flammability : 1 / 1 Instability : 0 / 0 Other :

0 = Insignificant 1 = Slight 2 = Moderate 3 = High 4 = Extreme

2. Composition/Information on Ingredients

Our hazard evaluation has identified the following chemical substance(s) as hazardous. Consult Section 15 for the

Nature of the hazard(s).

Hazardous Substance(s) CAS NO % (w/w)

Distillates, petroleum, hydro treated light 64742-47-8 10.0 - 30.0

Propylene Glycol 57-55-6 1.0 - 5.0

Organic sulfonic acid salt Proprietary 10.0 - 30.0

3. Hazards Identification

****EMERGENCY OVERVIEW****

WARNING

Combustible.

Keep away from heat. Keep away from sources of ignition - No smoking. Keep container tightly closed. Do not get in eyes, on skin, on clothing.

Do not take internally.

Avoid breathing vapor.

Use with adequate ventilation.

In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. After contact with skin, wash immediately with plenty of soap and water.

Wear suitable protective clothing.

Low Fire Hazard; liquids may burn upon heating to temperatures at or above the flash point. May evolve oxides of

Carbon (COx) under fire conditions. May evolve oxides of sulfur (SOx) under fire conditions.

Primary Routes of Exposure:

Eye, Skin

Human Health Hazards - Acute:

Eye Contact: May cause irritation with prolonged contact.

Skin Contact: May cause irritation with prolonged contact.

Ingestion: Not a likely route of exposure. Can cause chemical pneumonia if aspirated into lungs following ingestion.

Inhalation: Repeated or prolonged exposure may irritate the respiratory tract.

Symptoms of Exposure:

Acute: A review of available data does not identify any symptoms from exposure not previously mentioned.

Chronic: Frequent or prolonged contact with product may defect and dry the skin, leading to discomfort and dermatitis.

Aggravation of Existing Conditions: Skin contact may aggravate an existing dermatitis condition.

4. First Aid Measures

Eye Contact: Immediately flush with plenty of water for at least 15 minutes. If symptoms develop, seek medical advice.

Skin Contact: Immediately wash with plenty of soap and water. If symptoms develop, seek medical advice.

Ingestion: Do not induce vomiting: contains petroleum distillates and/or aromatic solvents. If conscious, washout mouth and give water to drink. Get medical attention.

Inhalation: Remove to fresh air, treat symptomatically. Get medical attention.

Note to Physician: Based on the individual reactions of the patient, the physician's judgment should be used to control symptoms and clinical condition.

5. Fire Fighting Measures

Flash Point: 181.4 °F / 83 °C (PMCC)

Lower Explosion Limit: Not flammable

Upper Explosion Limit: Not flammable

Extinguishing Media: Alcohol foam, Carbon dioxide, Foam, Dry powder, other extinguishing agent suitable for Class B fires, for large fires, use water spray or fog, thoroughly drenching the burning material. Water mist may be used to cool closed containers.

Unsuitable Extinguishing Media: Do not use water unless flooding amounts are available.

Fire and Explosion Hazard: Low Fire Hazard; liquids may burn upon heating to temperatures at or above the flash point. May evolve oxides of carbon (COx) under fire conditions. May evolve oxides of sulfur (SOx) under fire conditions.

Special Protective Equipment for Fire Fighting: In case of fire, wear a full face positive-pressure self contained breathing apparatus and protective suit.

6. Accidental Release Measures

Personal Precautions: Restrict access to area as appropriate until clean-up operations are complete. Stop or reduce any leaks if it is safe to do so. Ventilate spill area if possible. Do not touch spilled material. Remove sources of ignition. Have emergency equipment (for fires, spills, leaks, etc.) readily available. Use personal protective equipment recommended in Section 8 (Exposure Controls/Personal Protection). Notify appropriate government, occupational health and safety and environmental authorities.

Methods for Cleaning Up:

Small Spills: Soak up spill with absorbent material. Place residues in a suitable, covered, properly labeled container. Wash affected area. LARGE SPILLS: Contain liquid using absorbent material, by digging trenches or by diking. Reclaim into recovery or salvage drums or tank truck for proper disposal. Clean contaminated surfaces with water or aqueous cleaning agents. Contact an approved waste hauler for disposal of contaminated recovered material. Dispose of material in compliance with regulations indicated in Section 13 (Disposal Considerations).

Environmental Precautions: Do not contaminate surface water.

7. Handling and Storage

Handling: Use with adequate ventilation. Keep the containers closed when not in use. Do not take internally. Do not get in eyes, on skin, on clothing. Have emergency equipment (for fires, spills, leaks, etc.) readily available.

Storage Condition: Store away from heat and sources of ignition. Store separately from oxidizers. Store the containers tightly closed.

Suitable Construction Material: Compatibility with plastic materials can vary; we therefore recommend that compatibility is tested prior to use.

8. Exposure Controls/Personal Protection

Occupational Exposure Limits: Exposure guidelines have not been established for this product. Available exposure limits for the substance(s) are shown below:

ACGIH/TLV:

Oil Mist TWA: 5 mg/m3

STEL: 10 mg/m3

Propylene Glycol

OSHA/PEL:

Oil Mist TWA: 5 mg/m3

STEL: 10 mg/m3

Propylene Glycol

AIHA/WEEL:

Engineering Measures: General ventilation is recommended.

Respiratory Protection: Where concentrations in air may exceed the limits given in this section, the use of a half face filter mask or air supplied breathing apparatus is recommended. A suitable filter material depends on the amount and type of chemicals being handled. Consider the use of filter type: Multi-contaminant cartridge with a Particulate pre-filter. In event of emergency or planned entry into unknown concentrations a positive pressure, full-face piece SCBA should be used. If respiratory protection is required, institute a complete respiratory protection program including selection, fit testing, training, maintenance and inspection.

Hand Protection: Nitrile gloves, PVC gloves

Skin Protection: Wear standard protective clothing.

Eye Protection: Wear chemical splash goggles.

Hygiene Recommendations: Keep an eye wash fountain available. Keep a safety shower available. If clothing is contaminated, remove clothing and thoroughly wash the affected area. Launder contaminated clothing before reuse.

Human Exposure Characterization: Based on our recommended product application and personal protective equipment, the potential human exposures: Low.

9. Physical and Chemical Properties

Physical State: Liquid

Appearance: Clear Hazy Amber

Odor: Hydrocarbon

Specific Gravity: 0.95 @ 60 °F / 15.6 °C

Density: 7.91 lb/gal

Solubility in Water: Miscible

pH: (100 %) 6.2

Viscosity: 177 cps @ 32 °F / 0 °C 70 cps @ 60 °F / 15.6 °C @ 104 °F / 40 °C

Viscosity: @ 32 °F / 0 °C @ 60 °F / 15.6 °C 22.5 cst @ 104 °F / 40 °C

Pour Point: < -71 °F / < -57 °C

Boiling Point: 296 °F / 147 °C

Vapor Pressure: 15.5 mm Hg @ 100 °F / 37.8 °C

Note: These physical properties are typical values for this product and are subject to change.

10. Stability and Reactivity

Stability: Stable under normal conditions.

Hazardous Polymerization: Hazardous polymerization will not occur.

Conditions to Avoid: Heat

Materials to avoid: Contact with strong oxidizers (e.g. chlorine, peroxides, chromates, nitric acid, perchlorate, concentrated oxygen, permanganate) may generate heat, fires, explosions and/or toxic vapors.

Hazardous Decomposition Products: Under fire conditions, Oxides of carbon, Oxides of sulfur

11. Toxicological Information

No toxicity studies have been conducted on this product.

Sensitization: This product is not expected to be a sensitizer.

Carcinogenicity: None of the substances in this product are listed as carcinogens by the International Agency for Research on Cancer (IARC), the National Toxicology Program (NTP) or the American Conference of Governmental Industrial Hygienists (ACGIH).

Human Hazard Characterization: Based on our hazard characterization, the potential human hazard is: Moderate.

12. Analysis for Heavy Metals, Cyanide, and Chlorinated Hydrocarbons

Compound	**Concentration (ppm)**
Arsenic	0.16
Cadmium	N/D
Chromium	0.03
Copper	0.10
Lead	N/D
Mercury	N/D
Nickel	N/D
Zinc	N/D
Cyanide	N/D
Chlorinated Hydrocarbons	N/D

N/D = Not detected

13. Ecological Information

Ecotoxicological Effects:

The following results are for the product.

Acute Invertebrate Results:

Species Exposure LC_{50} EC_{50} Test Descriptor

Acartia tonsa 48 hrs 34 mg/l Product

Artemia 48 hrs 20.7 mg/l Product

Material Tested	**Species**	**LC_{50} (ppm)**
COREXIT® EC9500A	*Menidia beryllina*	25.20 96-hr
	Mysidopsis bahia	32.23 48-hr
No. 2 Fuel Oil	*Menidia beryllina*	10.72 96-hr
	Mysidopsis	16.12 48-hr

	bahia	
COREXIT® EC9500A & No. 2 Fuel Oil (1:10)	*Menidia beryllina*	2.61 96-hr
	Mysidopsis bahia	3.40 48-hr
Reference Toxicant (SDS)	*Menidia beryllina*	7.07 96-hr
	Mysidopsis bahia	9.82 48-hr

NOTE: This toxicity data was derived using the concentrated product. See Section VI of this bulletin for information regarding the manufacturer's recommendations for concentrations and application rates for field use.

Mobility: The environmental fate was estimated using a level III fugacity model embedded in the EPI (estimation program interface) Suite TM, provided by the US EPA. The model assumes a steady state condition between the total input and output. The level III model does not require equilibrium between the defined media. The information provided is intended to give the user a general estimate of the environmental fate of this product under the defined conditions of the models. If released into the environment, this material is expected to distribute to the air, water and soil/sediment in the approximate respective percentages.
Air Water Soil/Sediment <5% 10 - 30% 50 - 70%.
The portion in water is expected to float on the surface.
Bioaccumulation Potential: Component substances have a potential to bioconcentrate.
Environmental Hazard and Exposure Characterization: Based on our hazard characterization, the potential environmental hazard is: Low.
Based on our recommended product application and the product's characteristics, the potential environmental exposure is: Low.
If released into the environment, see CERCLA/SUPERFUND in Section 1.

14. Disposal Considerations

If this product becomes a waste, it could meet the criteria of a hazardous waste as defined by the Resource. Conservation and Recovery Act (RCRA) 40 CFR 261. Before disposal, it should be determined if the waste meets the criteria of a hazardous waste.
Hazardous Waste: D018
Hazardous wastes must be transported by a licensed hazardous waste transporter and disposed of or treated in a properly licensed hazardous waste treatment, storage, and disposal or recycling facility. Consult local, state, and federal regulations for specific requirements.

15. Transport Information

The information in this section is for reference only and should not take the place of a shipping paper (bill of lading) specific to an order. Please note that the proper Shipping Name / Hazard Class may vary by packaging, properties, and mode of transportation.
Typical Proper Shipping Names for this product are as follows.
Land Transport: For Packages Less Than or Equal To 119 Gallons:
Proper Shipping Name: PRODUCT IS NOT REGULATED DURING TRANSPORTATION.
For Packages Greater Than 119 Gallons:
Proper Shipping Name: COMBUSTIBLE LIQUID, N.O.S.
Technical Name(s): PETROLEUM DISTILLATES
UN/ID No: NA 1993
Hazard Class - Primary: COMBUSTIBLE
Packing Group: III
Flash Point: 83 °C / 181.4 °F
AIR TRANSPORT (ICAO/IATA):
Proper Shipping Name: PRODUCT IS NOT REGULATED DURING TRANSPORTATION
MARINE TRANSPORT (IMDG/IMO):
Proper Shipping Name: PRODUCT IS NOT REGULATED DURING TRANSPORTATION

15. Regulatory Information

NATIONAL REGULATIONS, USA:
OSHA HAZARD COMMUNICATION RULE, 29 CFR 1910.1200:
Based on our hazard evaluation, the following substance(s) in this product is/are hazardous and the reason(s) is/are shown below.

Distillates, petroleum, hydrotreated light: Irritant

Propylene Glycol: Exposure Limit, Eye irritant

Organic sulfonic acid salt: Irritant

CERCLA/SUPERFUND, 40 CFR 117, 302:

Notification of spills of this product is not required.

SARA/SUPERFUND AMENDMENTS AND REAUTHORIZATION ACT OF 1986 (TITLE III) - SECTIONS 302, 311,

312, AND 313:

SECTION 302 - EXTREMELY HAZARDOUS SUBSTANCES (40 CFR 355):

This product does not contain substances listed in Appendix A and B as an Extremely Hazardous Substance.

SECTIONS 311 AND 312 - MATERIAL SAFETY DATA SHEET REQUIREMENTS (40 CFR 370): Our hazard evaluation has found this product to be hazardous. The product should be reported under the following indicated EPA hazard categories:

X Immediate (Acute) Health Hazard

- Delayed (Chronic) Health Hazard

- Fire Hazard

- Sudden Release of Pressure Hazard

- Reactive Hazard

Under SARA 311 and 312, the EPA has established threshold quantities for the reporting of hazardous chemicals.

The current thresholds are: 500 pounds or the threshold planning quantity (TPQ), whichever is lower, for extremely hazardous substances and 10,000 pounds for all other hazardous chemicals.

SECTION 313 - LIST OF TOXIC CHEMICALS (40 CFR 372):

This product does not contain substances on the List of Toxic Chemicals.

TOXIC SUBSTANCES CONTROL ACT (TSCA):

The substances in this preparation are included on or exempted from the TSCA 8(b) Inventory (40 CFR 710)

FEDERAL WATER POLLUTION CONTROL ACT, CLEAN WATER ACT, 40 CFR 401.15 / formerly Sec. 307, 40

CFR 116.4 / formerly Sec. 311:

None of the substances are specifically listed in the regulation.

CLEAN AIR ACT, Sec. 111 (40 CFR 60, Volatile Organic Compounds), Sec. 112 (40 CFR 61, Hazardous Air Pollutants), Sec. 602 (40 CFR 82, Class I and II Ozone Depleting Substances): None of the substances are specifically listed in the regulation.
Substance(s) Citations

• Propylene Glycol Sec. 111

CALIFORNIA PROPOSITION 65:

This product does not contain substances which require warning under California Proposition 65.

MICHIGAN CRITICAL MATERIALS:

None of the substances are specifically listed in the regulation.

STATE RIGHT TO KNOW LAWS:

The following substances are disclosed for compliance with State Right to Know Laws:
Propylene Glycol 57-55-6

NATIONAL REGULATIONS, CANADA:

WORKPLACE HAZARDOUS MATERIALS INFORMATION SYSTEM (WHMIS):

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations
(CPR) and the MSDS contain all the information required by the CPR.

WHMIS CLASSIFICATION:

Not considered a WHMIS controlled product.

CANADIAN ENVIRONMENTAL PROTECTION ACT (CEPA):

The substances in this preparation are listed on the Domestic Substances List (DSL), are exempt, or have been reported in accordance with the New Substances Notification Regulations.

16. Other Information

Due to our commitment to Product Stewardship, we have evaluated the human and environmental hazards and exposures of this product. Based on our recommended use of this product, we have characterized the product's
general risk. This information should provide assistance for your own risk management practices. We have evaluated our product's risk as follows:

* The human risk is: Low

* The environmental risk is: Low

Any use inconsistent with our recommendations may affect the risk characterization. Our sales representative will assist you to determine if your product application is

consistent with our recommendations. Together we can implement an appropriate risk management process.

This product material safety data sheet provides health and safety information. The product is to be used in applications consistent with our product literature. Individuals handling this product should be informed of the recommended safety precautions and should have access to this information. For any other uses, exposures should be evaluated so that appropriate handling practices and training programs can be established to insure safe workplace operations. Please consult your local sales representative for any further information.

17. References

Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices, American Conference of Governmental Industrial Hygienists, OH., (Ariel Insight# CD-ROM Version), Ariel Research Corp., Bethesda, MD.

Hazardous Substances Data Bank, National Library of Medicine, Bethesda, Maryland (TOMES CPS# CD-ROM Version), Micromedex, Inc., Englewood, CO.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Geneva: World Health Organization, International Agency for Research on Cancer.

Integrated Risk Information System, U.S. Environmental Protection Agency, Washington, D.C. (TOMES CPS# CDROM Version), Micromedex, Inc., Englewood, CO.

Annual Report on Carcinogens, National Toxicology Program, U.S. Department of Health and Human Services, Public Health Service.

Title 29 Code of Federal Regulations, Part 1910, Subpart Z, Toxic and Hazardous Substances, Occupational Safety and Health Administration (OSHA), (Ariel Insight# CD-ROM Version), Ariel Research Corp., Bethesda, MD.

Registry of Toxic Effects of Chemical Substances, National Institute for Occupational Safety and Health, Cincinnati, OH, (TOMES CPS# CD-ROM Version), Micromedex, Inc., Englewood, CO.

Ariel Insight# (An integrated guide to industrial chemicals covered under major regulatory and advisory programs), North American Module, Western European Module, Chemical Inventories Module and the Generics Module (Ariel Insight# CD-ROM Version), Ariel Research Corp., Bethesda, MD.

The Teratogen Information System, University of Washington, Seattle, WA (TOMES CPS# CD-ROM Version),

Micromedex, Inc., Englewood, CO.

Prepared By: Product Safety Department

Date issued: 06/14/2005

Version Number: 1.6

(800) 424-9300 (24 Hours) CHEMTREC

From (USEPA, OIL PROGRAM CENTER, 1994; Nalco Energy Services, L.P, 2004).

Table A-2: Corexit® 9527 Material, Safety and Data Sheet

(800) 424-9300 (24 Hours) CHEMTREC

1. Chemical Product and Company Identification

Product Name: **COREXIT® 9527**

Application: Oil Spill Dispersant

Company Identification: Nalco Energy Services, L.P., P.O. Box 87, Sugar Land, Texas, 77487-0087

Emergency Telephone Numbers: (800) 424-9300 (24 Hours) CHEMTREC

NFPA 704M/HMIS RATING

Health : 2 / 2 Flammability : 2 / 2 Instability : 0 / 0 Other :

0 = Insignificant 1 = Slight 2 = Moderate 3 = High 4 = Extreme

2. Composition/Information on Ingredients

Our hazard evaluation has identified the following chemical substance(s) as hazardous. Consult Section 15 for the Nature of the hazard(s).

Hazardous Substance(s) CAS NO % (w/w)

2-Butoxyethanol 111-76-2 30.0 -60.0

Organic sulfonic acid salt Proprietary 10.0 -30.0

Propylene Glycol 57-55-6 1.0 -5.0

3. Hazards Identification

****EMERGENCY OVERVIEW****

WARNING

Combustible.

Eye and skin irritant. Repeated or excessive exposure to butoxyethanol may cause injury to red blood cells (hemolysis), kidney or the liver.

Combustible.

Do not get in eyes, on skin, on clothing. Do not take internally. Use with adequate ventilation. Wear suitable and protective clothing. Keep container tightly closed. Flush affected area with water. Keep away from heat. Keep away from sources of ignition - No smoking.

May evolve oxides of carbon (COx) under fire conditions.

Wear suitable protective clothing.

Low Fire Hazard; liquids may burn upon heating to temperatures at or above the flash point. May evolve oxides of carbon (COx) under fire conditions. May evolve oxides of sulfur (SOx) under fire conditions.

Primary Routes of Exposure:

Eye, Skin

Human Health Hazards - Acute:

Eye Contact: Can cause mild to moderate irritation

Skin Contact: Can cause mild to moderate irritation.

Ingestion: Not a likely route of exposure. Large quantities may cause kidney and liver damage.

Inhalation: Not a likely route of exposure. Aerosols or product mist may irritate the upper respiratory tract.

Symptoms of Exposure:

Acute: Excessive exposure may cause central nervous system effects, nausea, and vomiting, anesthetic or narcotic effects.

Chronic: Repeated or excessive exposure to butoxyethanol may cause injury to red blood cells (hemolysis), kidney or the liver.

Aggravation of Existing Conditions: Skin contact may aggravate an existing dermatitis condition.

4. First Aid Measures

Eye Contact: Flush affected area with water. If symptoms develop, seek medical advice.

Skin Contact: Flush affected area with water. If symptoms develop, seek medical advice.

Ingestion: Do not induce vomiting without medical advice. If conscious, washout mouth and give water to drink. If symptoms develop, seek medical advice.

Inhalation: Remove to fresh air, treat symptomatically. If symptoms develop, seek medical advice.

Note to Physician: Based on the individual reactions of the patient, the physician's judgment should be used to control symptoms and clinical condition.

5. Fire Fighting Measures

Flash Point: 163 °F / 72.7 °C (TCC)

Extinguishing Media: This product would not be expected to burn unless all the water is boiled away. The remaining organics may be ignitable. Use extinguishing media appropriate for surrounding fire.

Fire and Explosion Hazard: May evolve oxides of carbon (COx) under fire conditions.

Special Protective Equipment for Fire Fighting: In case of fire, wear a full face positive-pressure self contained breathing apparatus and protective suit.

6. Accidental Release Measures

Personal Precautions: Restrict access to area as appropriate until clean-up operations are complete. Stop or reduce any leaks if it is safe to do so. Do not touch spilled material. Ventilate spill area if possible. Use personal protective equipment recommended in Section 8 (Exposure Controls/Personal Protection).

Methods for Cleaning Up:

Small Spills: Soak up spill with absorbent material. Place residues in a suitable, covered, properly labeled container. Wash affected area.

Large Spills: Contain liquid using absorbent material, by digging trenches or by diking. Reclaim into recovery or salvage drums or tank truck for proper disposal. Clean contaminated surfaces with water or aqueous cleaning agents. Contact an approved waste hauler for disposal of contaminated recovered material. Dispose of material in compliance with regulations indicated in Section 13 (Disposal Considerations).

Environmental Precautions: Do not contaminate surface water.

7. Handling and Storage

Handling: Avoid eye and skin contact. Do not take internally. Ensure all containers are labeled. Keep the containers closed when not in use.

Storage Condition: Store the containers tightly closed.

Suitable Construction Material: PVC, Stainless Steel 316L, Hastelloy C-276, MDPE (medium density polyethylene), Nitrile, Plexiglass, Kalrez, EPDM, TFE, Alfax, Teflon, HDPE (high density polyethylene), Neoprene, Aluminum, Polypropylene, Polyethylene, Carbon Steel C1018, Stainless Steel 304, Compatibility with Plastic Materials can vary; we therefore recommend that compatibility is tested prior to use.

Unsuitable Construction Material: Copper, Mild steel, Brass, Nylon, Buna-N, Natural rubber, Polyurethane, Hypalon, Viton, Ethylene propylene.

8. Exposure Controls/Personal Protection

Occupational Exposure Limits: Exposure guidelines have not been established for this product. Available exposure limits for the substance(s) are shown below:
ACGIH/TLV: 2-Butoxyethanol TWA: 20 ppm, 97 mg/m3
Propylene Glycol
OSHA/PEL: Substance(s) 2-Butoxyethanol TWA: 25 ppm, 120 mg/m3 (Skin).
Propylene Glycol
AIHA/WEEL: Substance(s) for propylene glycol, an 8 hour TWA of 10 mg/m3 (aerosol) and 50 ppm (total).

Engineering Measures: General ventilation is recommended.

Respiratory Protection: Where concentrations in air may exceed the limits given in this section, the use of a half face filter mask or air supplied breathing apparatus is recommended. A suitable filter material depends on the amount and type of chemicals being handled. Consider the use of filter type: Multi-contaminant cartridge (Gold) with a Particulate pre-filter (Purple). In event of emergency or planned entry into unknown concentrations a positive pressure, full face piece SCBA should be used. If respiratory protection is required, institute a complete respiratory protection program including selection, fit testing, training, maintenance and inspection.

Hand Protection: Nitrile gloves, PVC gloves

Skin Protection: Wear standard protective clothing.

Eye Protection: Wear chemical splash goggles.

Hygiene Recommendations: Keep an eye wash fountain available. Keep a safety shower available. If clothing is contaminated, remove clothing and thoroughly wash the affected area. Launder contaminated clothing before reuse.

Human Exposure Characterization: Based on our recommended product application and personal protective equipment, the potential human exposures: Low.

9. Physical and Chemical Properties

Physical State: Liquid

Appearance: Clear Amber

Odor: Mild

Specific Gravity: 0.98 - 1.02

Density: 8.2 - 8.5 lb/gal

Solubility in Water: Complete

pH: (100 %) 6.1

Viscosity: 160 cst @ 32 °F / 0 °C

Pour Point: < -40 °F / < -40 °C

Boiling Point: 340 °F / 171 °C

Vapor Pressure: < 5 mm Hg @ 100 °F / 38 °C same as water

Evaporation Rate: 0.1

Note: These physical properties are typical values for this product and are subject to change.

10. Stability and Reactivity

Stability: Stable under normal conditions.

Hazardous Polymerization: Hazardous polymerization will not occur.

Conditions to avoid: Freezing temperatures.

Materials to avoid: None known

Hazardous Decomposition Products: Under fire conditions: Oxides of carbon.

11. Toxicological Information

No toxicity studies have been conducted on this product.

Sensitization: This product is not expected to be a sensitizer.

Carcinogenicity: None of the substances in this product are listed as carcinogens by the International Agency for Research on Cancer (IARC), the National Toxicology Program (NTP) or the American Conference of Governmental Industrial Hygienists (ACGIH).

Human Hazard Characterization: Based on our hazard characterization, the potential human hazard is: High.

12. Analysis for Heavy Metals, Cyanide, and Chlorinated Hydrocarbons

Compound	**Concentration (ppm)**
Arsenic	0.16
Cadmium	N/D
Chromium	0.03
Copper	0.10
Lead	N/D
Mercury	N/D
Nickel	N/D
Zinc	N/D
Cyanide	N/D

Chlorinated Hydrocarbons	N/D

N/D = Not detected

13. Ecological Information

Ecotoxicological Effects:

No toxicity studies have been conducted on this product.

Acute Fish Results:

Species Exposure LC_{50} Test Descriptor

Turbot 96 hrs 50 mg/l

Mobility: The environmental fate was estimated using a level III fugacity model embedded in the EPI (estimation program interface) Suite TM, provided by the US EPA. The model assumes a steady state condition between the total input and output. The level III model does not require equilibrium between the defined media. The information provided is intended to give the user a general estimate of the environmental fate of this product under the defined conditions of the models. If released into the environment this material is expected to distribute to the air, water and soil/sediment in the approximate respective percentages; Air Water Soil/Sediment <5% 10 - 30% 70 - 90%

The portion in water is expected to be soluble or dispersible. The portion in water is expected to float on the surface.

Bioaccumulation Potential: Component substances have a low potential to bioconcentrate.

Environmental Hazard and Exposure Characterization: Based on our hazard characterization, the potential environmental hazard is: Moderate

Based on our recommended product application and the product's characteristics, the potential environmental exposure is: Low

If released into the environment, see CERCLA/SUPERFUND in Section 15.

14. Disposal Considerations

If this product becomes a waste, it is not a hazardous waste as defined by the Resource Conservation and Recovery.

Act (RCRA) 40 CFR 261, since it does not have the characteristics of Subpart C, nor is it listed under Subpart D.

As a non-hazardous waste, it is not subject to federal regulation. Consult state or local regulation for any additional handling, treatment or disposal requirements. For disposal, contact a properly licensed waste treatment, storage, disposal or recycling facility.

15. Transport Information

The information in this section is for reference only and should not take the place of a shipping paper (bill of lading) specific to an order. Please note that the proper Shipping Name / Hazard Class may vary by packaging, properties, and mode of transportation.

Typical Proper Shipping Names for this product are as follows.

Land Transport: For Packages Less Than or Equal To 119 Gallons:

Proper Shipping Name: PRODUCT IS NOT REGULATED DURING TRANSPORTATION.

For Packages Greater Than 119 Gallons:

Proper Shipping Name:

Technical Name(s):

UN/ID No:

Hazard Class - Primary:

Packing Group:

COMBUSTIBLE LIQUID, N.O.S.

2-BUTOXYETHANOL

NA 1993

COMBUSTIBLE III

Flash Point: 72.7 °C / 163 °F

AIR TRANSPORT (ICAO/IATA):

Proper Shipping Name: PRODUCT IS NOT REGULATED DURING TRANSPORTATION

MARINE TRANSPORT (IMDG/IMO):

Proper Shipping Name: PRODUCT IS NOT REGULATED DURING TRANSPORTATION

15. Regulatory Information

NATIONAL REGULATIONS, USA:

OSHA HAZARD COMMUNICATION RULE, 29 CFR 1910.1200:

Based on our hazard evaluation, none of the substances in this product are hazardous.

CERCLA/SUPERFUND, 40 CFR 117, 302:

Notification of spills of this product is not required.

SARA/SUPERFUND AMENDMENTS AND REAUTHORIZATION ACT OF 1986 (TITLE III) - SECTIONS 302, 311,
312, AND 313:
SECTION 302 - EXTREMELY HAZARDOUS SUBSTANCES (40 CFR 355):
This product does not contain substances listed in Appendix A and B as an Extremely Hazardous Substance.
SECTIONS 311 AND 312 - MATERIAL SAFETY DATA SHEET REQUIREMENTS (40 CFR 370):
Our hazard evaluation has found this product to be hazardous. The product should be reported under the following indicated EPA hazard categories:
X Immediate (Acute) Health Hazard
X Delayed (Chronic) Health Hazard
X Fire Hazard
Sudden Release of Pressure Hazard
Reactive Hazard
Under SARA 311 and 312, the EPA has established threshold quantities for the reporting of hazardous chemicals.
The current thresholds are: 500 pounds or the threshold planning quantity (TPQ), whichever is lower, for extremely hazardous substances and 10,000 pounds for all other hazardous chemicals.
SECTION 313 - LIST OF TOXIC CHEMICALS (40 CFR 372):
This product contains the following substance(s), (with CAS # and % range) which appear(s) on the List of Toxic Chemicals.
Hazardous Substance(s)
Glycol Ethers
CAS NO % (w/w)
0.0 - 0.0
TOXIC SUBSTANCES CONTROL ACT (TSCA):
The substances in this preparation are included on or exempted from the TSCA 8(b) Inventory (40 CFR 710)
FEDERAL WATER POLLUTION CONTROL ACT, CLEAN WATER ACT, 40 CFR 401.15 / formerly Sec. 307, 40
CFR 116.4 / formerly Sec. 311:
None of the substances are specifically listed in the regulation.

CLEAN AIR ACT, Sec. 111 (40 CFR 60, Volatile Organic Compounds), Sec. 112 (40 CFR 61, Hazardous Air

Pollutants), Sec. 602 (40 CFR 82, Class I and II Ozone Depleting Substances):

This product contains the following substances listed in the regulation:

Substance(s) Citations

• 2-Butoxyethanol Sec. 111

• Propylene Glycol

CALIFORNIA PROPOSITION 65:

This product does not contain substances which require warning under California Proposition 65.

MICHIGAN CRITICAL MATERIALS:

None of the substances are specifically listed in the regulation.

STATE RIGHT TO KNOW LAWS:

The following substances are disclosed for compliance with State Right to Know Laws:

2-Butoxyethanol 111-76-2

Propylene Glycol 57-55-6

NATIONAL REGULATIONS, CANADA:

WORKPLACE HAZARDOUS MATERIALS INFORMATION SYSTEM (WHMIS):

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations (CPR) and the MSDS contains all the information required by the CPR.

WHMIS CLASSIFICATION:

D2B - Materials Causing Other Toxic Effects - Toxic Material

CANADIAN ENVIRONMENTAL PROTECTION ACT (CEPA):

The substances in this preparation are listed on the Domestic Substances List (DSL), are exempt, or have been reported in accordance with the New Substances Notification Regulations.

16. Other Information

Due to our commitment to Product Stewardship, we have evaluated the human and environmental hazards and exposures of this product. Based on our recommended use of this product, we have characterized the product's general risk. This information should provide assistance for your own risk management practices. We have evaluated our product's risk as follows:

* The human risk is: Low

* The environmental risk is: Low

Any use inconsistent with our recommendations may affect the risk characterization. Our sales representative will assist you to determine if your product application is consistent with our recommendations. Together we can implement an appropriate risk management process.

This product material safety data sheet provides health and safety information. The product is to be used in applications consistent with our product literature. Individuals handling this product should be informed of the recommended safety precautions and should have access to this information. For any other uses, exposures should be evaluated so that appropriate handling practices and training programs can be established to insure safe workplace operations. Please consult your local sales representative for any further information.

17. References

Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices, American Conference of Governmental Industrial Hygienists, OH., (Ariel Insight# CD-ROM Version), Ariel Research Corp., Bethesda, MD.

Hazardous Substances Data Bank, National Library of Medicine, Bethesda, Maryland (TOMES CPS# CD-ROM Version), Micromedex, Inc., Englewood, CO.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Geneva: World Health Organization, International Agency for Research on Cancer.

Integrated Risk Information System, U.S. Environmental Protection Agency, Washington, D.C. (TOMES CPS# CDROM Version), Micromedex, Inc., Englewood, CO.

Annual Report on Carcinogens, National Toxicology Program, U.S. Department of Health and Human Services, Public Health Service.

Title 29 Code of Federal Regulations, Part 1910, Subpart Z, Toxic and Hazardous Substances, Occupational Safety and Health Administration (OSHA), (Ariel Insight# CD-ROM Version), Ariel Research Corp., Bethesda, MD.

Registry of Toxic Effects of Chemical Substances, National Institute for Occupational Safety and Health, Cincinnati, OH, (TOMES CPS# CD-ROM Version), Micromedex, Inc., Englewood, CO.

Ariel Insight# (An integrated guide to industrial chemicals covered under major regulatory and advisory programs), North American Module, Western European

Module, Chemical Inventories Module and the Generics Module (Ariel Insight# CD-ROM Version), Ariel Research Corp., Bethesda, MD.

The Teratogen Information System, University of Washington, Seattle, WA (TOMES CPS# CD-ROM Version), Micromedex, Inc., Englewood, CO.

Prepared By: Product Safety Department

Date issued: 02/20/2004

Version Number: 1.6

(800) 424-9300 (24 Hours) CHEMTREC

From (**Nalco Energy Services, L.P, 2004).**

Table A-3: Slickgone NS® Material, Safety and Data Sheet

DASIC
INTERNATIONAL LTD

Slickgone NS

Reference F.315

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REVISION 2
ISSUE DATE 15-11-1995

14.TRANSPORT INFORMATION (REGULATIONS)

PACKAGING (Size & Description)	200 or 25 litre steel containers. 1000 litre intermediate bulk containers with fluorinated polythene liners.
TRANSPORT CLASSIFICATION	
SUBSTANCE IDENTIFICATION NUMBER	-
PROPER SHIPPING NAME	
ICAO/IATA/IMDG CLASS	-
SUBSIDIARY RISK	-
PACKING GROUP	-
ADR CLASS	-
TRANSPORT HAZARD SYMBOL	
HAZARD IDENT NUMBER	-
EMERGENCY ACTION CODE	-
OTHER	Not classified as hazardous under IMO, IATA or UK transport regulations.

15.REGULATORY INFORMATION (Supply & Labelling)

SUPPLY CLASSIFICATION	Not Regulated.
HAZARD PICTOGRAM	
RISK PHRASES	
SAFETY PHRASES	
OTHER APPLICABLE REGULATIONS	

16.OTHER INFORMATION

Use only in accordance with Dasic's use instructions. Do not use for other applications without first consulting the Dasic Technical Department for advice. The information contained in this safety data sheet is provided in accordance with the requirements of the Chemicals (Hazard Information and Packaging) Regulations.

REVISION 2 ISSUE DATE 15-11-1995

The information provided in this Safety Data Sheet is correct to the best of our knowledge at the date of issue. It is intended as a guide for safe handling, storage and use in known applications. References to regulatory matters are not intended to be exhaustive and the user must satisfy himself that all relevant legislation is complied with. This Safety Data Sheet should not be construed as a specification or guarantee of specific properties and no liability can be accepted for any loss, injury or damage resulting from its use.

DASIC INTERNATIONAL LIMITED
Winchester Hill · Romsey · Hampshire SO51 7YD · England
Tel: Romsey (01794) 512419 · *Fax:* Romsey (01794) 522346

Tel: Romsey (01794) 512419 · *Fax:* Romsey (01794) 522346 which may be made or that any use will not infringe any patent.

REVISION
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5.FIRE FI

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Name

DASIC

INTERNATIONAL LTD

Slickgone NS

Reference F.315

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REVISION 2
ISSUE DATE 15-11-1995

ENGINEERING MEASURES	No occupational exposure limit has been assigned by UK HSE or ACGIH to the solvent component of this product. However, the mineral oil mist figures of 5 mg/m3 8hr LTEL TWA and 10 mg/m3 15 min STEL, given in EH40 as OES values, are recommended. For the vapour, an occupational exposure limit of 200 ppm (1500 mg/m3) 8hr TWA is recommended. In areas of good ventilation and under normal conditions of use, it is unlikely that these values will be exceeded. If adequate ventilation cannot be supplied, a suitable organic cartridge respirator or self contained breathing apparatus should be used.
PERSONAL PROTECTIVE EQUIPMENT	
RESPIRATORY	Not normally required but see Engineering Measures above.
HAND	Wear impervious gloves.
EYE	Eye protection designed to protect against liquid splashes should be worn. Eye wash facilities should be available in areas where accidental exposure may be possible.
OTHER	Cotton or cotton/synthetic overalls or coveralls are normally suitable. Grossly contaminated clothing should be removed and the skin washed with soap and water or a proprietary skin cleaner.

9.PHYSICAL/CHEMICAL PROPERTIES

APPEARANCE	Clear, brown, slightly viscous liquid.
ODOUR	Mild and characteristic.
pH (as delivered)	Neutral
VISCOSITY	30 - 60 cP @ 0°C
FREEZING POINT	< -10°C
BOILING POINT (or RANGE)	Comm at 192°C
FLASH POINT	72°C PMCC
AUTOFLAMMABILITY	230°C
EXPLOSIVE LIMITS	
UPPER LIMIT	7.0
LOWER LIMIT	0.6
VAPOUR PRESSURE	≈ 0.04kPa @ 20°C
VAPOUR DENSITY	7.6 g/l @ 20°C
RELATIVE DENSITY (SG)	0.86 @ 20°C
SOLUBILITY	Disperses in water to form unstable emulsion.

10.STABILITY AND REACTIVITY

STABILITY	Stable at normal temperatures and pressures.
CONDITIONS TO AVOID	Avoid ignition sources. Do not heat material.
MATERIALS TO AVOID	Reacts violently with oxidising agents and concentrated nitric acid.
HAZARDOUS DECOMPOSITION PRODUCTS	None unusual. Burning will produce smoke, carbon monoxide, carbon dioxide and traces of sulphur dioxide/trioxide.

DASIC II
Winchester Hill
Tel: Romsey (01

DASIC INTERNATIONAL LIMITED
Winchester Hill · Romsey · Hampshire SO51 7YD · England
Tel: Romsey (01794) 512419 · *Fax:* Romsey (01794) 522346

DASIC
INTERNATIONAL LTD

Slickgone NS

REVISION 2
ISSUE DATE 15-11-1995

Reference F.315
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11.TOXICOLOGICAL INFORMATION

ACUTE EFFECTS

EYES Liquid splashes in the eye may cause irritation. High concentrations of vapour may also cause irritation.

SKIN Will cause defatting of skin which may lead to irritation and dermatitis on prolonged contact.

INGESTION May cause vomiting, nausea, central nervous system depression and diarrhoea. Aspiration during swallowing or vomiting will severely damage lungs.

INHALATION Vapour is irritant in high concentrations and may cause drowsiness, nausea, dizziness and possibly unconsciousness. This is unlikely to occur when the product is used to treat oil spills in the open sea.

CHRONIC EFFECTS

12.ECOLOGICAL INFORMATION

MOBILITY

PERSISTENCE & DEGRADABILITY The surfactant component of this product is 95% biodegradable when tested to EEC Directive 73/405/EEC as amended by 82/243/EEC. The whole product has been tested for ultimate biodegradability in the marine environment and the results have been accepted by the French government, as part of their approval procedure for dispersants.

BIO-ACCUMULATION Not expected to bio-accumulate.

AQUATIC TOXICITY This product has been tested for marine toxicity by the UK Ministry of Agriculture Fisheries and Foods and is approved as a Type 3 oil dispersant concentrate. It has also been tested and approved as a marine oil spill dispersant by the French goverrment laboratory "CEDRE".

OTHER

13.DISPOSAL CONSIDERATIONS

The material is classed as hazardous waste and must be disposed of via a licensed hazardous waste contractor, in accordance with the regulations prevailing in the locality/country concerned. Empty containers should be treated as hazardous waste. Do not dump indiscriminately.

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