Use of Rainbow Trout Liver Cell Line (RTL-W1) to evaluate the toxicity of Heavy Fuel Oil 7102

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

A rainbow trout liver cell line, RTL-W1, was used to evaluate the toxic potential of a heavy fuel oil (HFO) HFO 7102, and its fractions, which together with the HFO are referred to as the oil samples. The fractions were F2, F3, F3-1, F3-2 and F4 and had been prepared by low-temperature vacuum distillation by collaborators at Queen's University. For presentation to the cells, HFO 7102 and its fractions were made into High Energy-Chemically Enhanced Water Accommodated Fractions (HE-CEWAFs). The procedure for this involved adding Corexit 9500 to the oil samples, mixing them on a vortex, and letting the phases settle. The HE-CEWAFs were added to RTL-W1 cell cultures, and at various times afterwards cell viability and CYP1A induction were monitored.

Cell viability was evaluated with two dyes, Alamar Blue, which monitors energy metabolism, and 5-carboxfluorescein diacetate acetoxymethyl ester (CFDA AM), which measures plasma membrane integrity. With both indicator dyes, Corexit 9500 was cytotoxic but the concentrations eliciting cytotoxicity varied with the cell culture media. In Leibovitz's L-15 with fetal bovine serum (FBS), which was the medium used for studying CYP1A induction, Corexit 9500 was only cytotoxic at concentrations of 0.1 % (v/v) and greater. For the oil samples, F3-2 at 1 mg/ml and F4 at 10 mg/ml, which were the highest testable concentrations for each, no loss of cell viability was observed over 24 h. The other oil samples were cytotoxic only at their highest testable concentrations, which ended being between 1 and 10 mg/ml.

CYP1A induction was monitored in RTL-W1 as catalytic activity and as the level of CYP1A (P4501A) protein. The catalytic activity was assayed as 7-ethoxyresorufin o-deethylase (EROD) activity; the CYP1A protein level, by western blotting. The positive control was 2, 3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which strongly induced both EROD activity and CYP1A

protein. Corexit 9500 by itself neither induced EROD activity nor CYP1A protein. All the oil samples induced both EROD activity and CYP1A protein. With both endpoints, the most potent fraction was F3; the least potent, F3-2. As the induction of CYP1A is associated with the development of blue sac disease (BSD) and mortality in early life stages of fish, the results suggest that HFO 7102 and its fractions have the potential to reduce recruitment of young into adult fish populations.

CYP1A induction by F3 was studied further, again through EROD activity and western blotting. As the F3 concentration was increased, EROD activity increased but declined at high concentrations, whereas CYP1A protein continued to increase. This suggests the presence of compounds in F3 that at high concentrations inhibit the catalytic activity of CYP1A. When F3 was presented to RTL-W1 cultures together with TCDD, CYP1A protein was induced but not EROD activity. Again this suggests that F3 contains inhibitor(s) of CYP1A as well as inducers. When cultures were exposed to either F3 or TCDD for 24 h and then followed by western blotting for up to 6 days after F3 or TCDD removal, CYP1A levels declined in F3 cultures but not in TCDD cultures. This suggests that RTL-W1 were able to inactivate CYP1A inducer(s) in F3 through metabolism. Overall the results suggest that the pattern of CYP1A induction by F3, and by extension, HFO involves complex interactions between the many chemical components in these mixtures. Likely the most important chemicals are the polycyclic aromatic hydrocarbons (PAHs).

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Acknowledgments

I have seen a lot of acknowledgements opened by sentences like "Wow, here I am" or "My head is spinning" and at this moment, I couldn't agree more. I can't believe that I could manage to finish this work one day! Of course, I have so many people to thanks. Without your help, I couldn't do this all by myself.

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List of Abbreviations

7 ER	7-ethoxyresorufin
AhR	Aryl hydrocarbon receptor
BaP	benzo[a]pyrene
BbF	benzo[b]fluorene
BkF	benzo[k]fluoranthene
BSD	Blue Sac disease
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
CYP 450	cytochrome P450
DBT	dibenzothiophene
DOSS	dioctyl sodium sulfosuccinate
EC_{50}	Median effective concentration
EDFA	Effects-Driven Fractionation and Analysis
EROD	7-ethoxyresorufin-o-deethylase
FBS	fetal bovine serum
Fl	fluoranthene
HE-CEWAF	High-energy chemically-enhanced water-accommodated fraction
HFO	Heavy fuel oil
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
RFU	raw florescent unites
ROS	reactive oxygen species
RTL-W1	Rainbow trout liver epithelial cell line
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TPAH	total petroleum aromatic hydrocarbon

Chapter 1 General introduction

1.1. Oil

The word 'oil' is often used very generally to refer to petroleum or any petroleum product. This is how 'oil' will be used in this document. However, when applicable, more specific descriptors will be used. Some of these are reviewed below.

1.1.1. Petroleum or crude oil

Petroleum or crude oil is a mixture of hydrocarbons and is found in geological formations, such as rock strata (Burger, 1997). Most petroleum is a fossil fuel. The petroleum started out as zooplankton and algae in shallow seas hundreds of millions of years ago. Over time, the remains of these organisms accumulated and were subjected to intense heat and pressure. Slowly the organic matter was transformed into petroleum/crude oil. This collected as subsurface pools in porous or fractured rock formations. These are known as petroleum reservoirs. Typically this crude oil can be extracted from the ground through oil wells. Thus what comes out of the ground is crude oil. A region with multiple oil wells is an oil field.

Crude oil has a complex chemistry. The number of different hydrocarbons in petroleum is very large. They can be grouped into four classes: alkanes (paraffins), cycloalkanes, aromatic hydrocarbons, and asphaltenes. Additionally, small amounts of other chemical compounds that contain sulphur, nitrogen and oxygen are also present (Wang et al., 2003).The composition varies with the geographical location of the oil field. Crude oil that flows easily is referred to as light crude oil, whereas crude oil that flows with difficulty is referred to as heavy crude oil. Crude oil is generally not used directly but must be refined.

1.1.2. Refining crude oil

Crude oil is refined to give industrial products, such as gasoline. Refining involves separating components through the process of heating and distillation. This is done in refineries and involves piping crude oil through hot furnaces. The liquids and vapours that result are discharged into distillation towers or columns (Figure 1.1). The hydrocarbons of crude oil have a wide range of boiling points. In the distillation tower, the liquids and vapours separate into components or fractions according to weight and boiling point (Figure 1.1). Liquid petroleum gas (small alkanes with 1 to 4 carbons), naphtha (5 to 9 carbon alkanes), and gasoline (alkanes and cycloalkanes of 5 to 12 carbons) are the lightest fractions and vaporize and rise to the top of the tower, where they condense back to liquids. Kerosene and diesel oil distillates are medium weight liquids and stay in the middle. The heaviest fraction with the highest boiling point is a tarlike fraction, called bitumen. Fuel oil separates between the medium weight liquids and bitumen. Fuel oil is the liquid or semi-liquid, high-boiling fraction of residue from the distillation of crude oil.
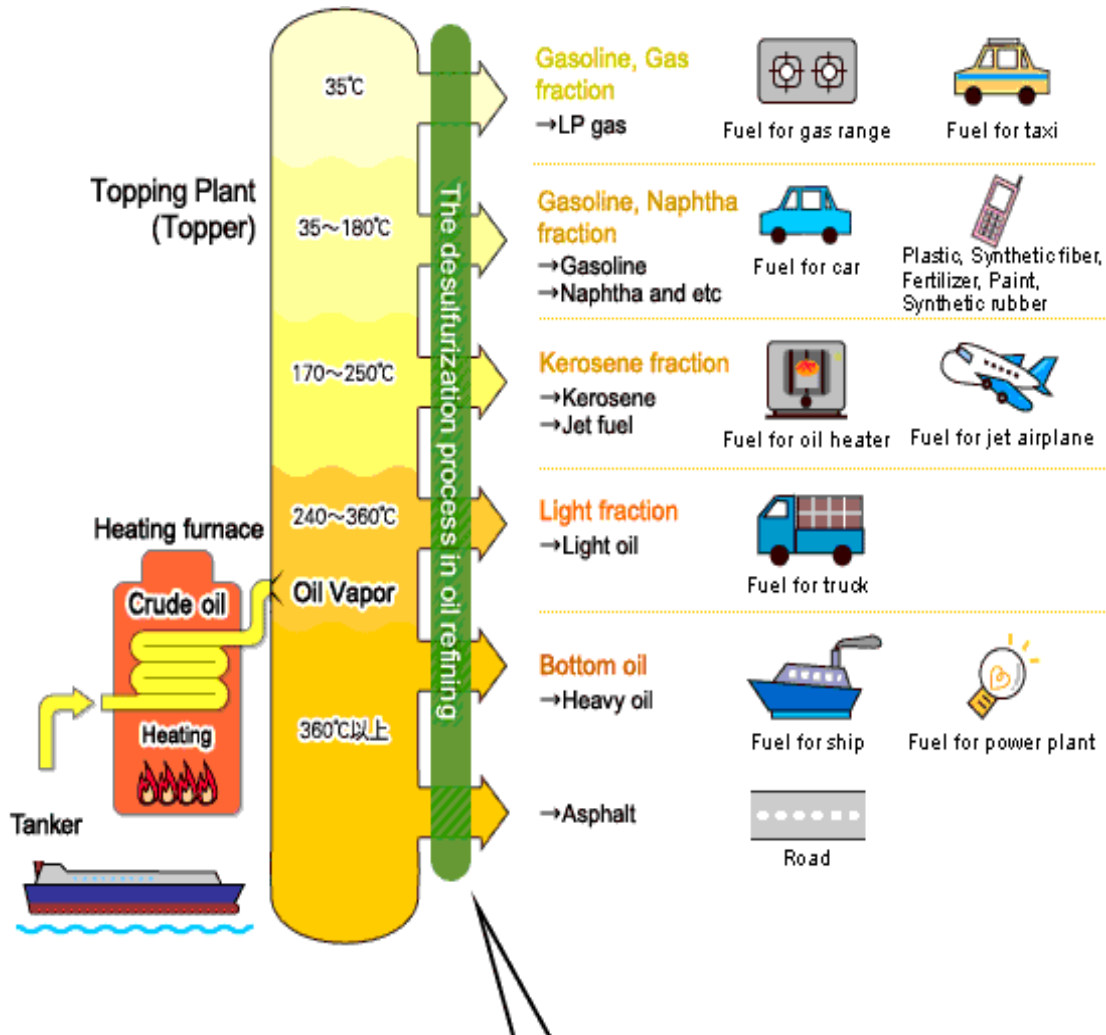
Figure 1.1. An overview of crude oil refining. *Obtained from the following web site http://tiptonceram.com/oil_refining/structure.html*

1.1.3. Fuel oil

Broadly speaking, fuel oil is the fractions that are obtained from petroleum distillation that are burned in a furnace or boiler to generate heat or used in engines to generate power. Some fuel oils are distillates. These include fuel oil numbers (No.) 1, 2, and 3. Sometimes these are termed diesel fuels, especially No. 2 fuel oil. No. 4, 5 and 6 are residual fuel oils. The descriptor residual is used because the more valuable fractions of crude oil have been boiled off. No. 5 and 6 are heavy fuel oils.

Heavy fuel oil is an important fuel for certain industries. Heavy fuel oil is burned in thermal-electric power plants and for heating boilers and furnaces in some manufacturing industries. These include pulp and paper plants and petroleum refineries. Heavy fuel oil is also used to heat some large, usually older, institutional and multiple residential buildings and to power engines of large marine vessels. Bunker B is often an alternative name for No 5. No. 6 is the subject of this thesis and is also known as Bunker C fuel oil.

1.1.4. Heavy fuel oil 7102 – Bunker C

Bunker C or Number 6 is a high viscosity heavy fuel oil (IARC, 1989). Trace minerals can be present if they were in the original crude oil but are concentrated as a result of the refining process. The hydrocarbon constituents dominate and have molecular weights ranging from about 300 to over 1000. They are complex mixtures that have been difficult to characterize in detail. Asphaltenes, resins (polar aromatics or heterocyclic aromatics), naphthalene aromatics, aromatics, and saturated hydrocarbons are some of the hydrocarbon classes (Neff and Anderson, 1981). Like other residual fuel oils, Bunker C has appreciable concentrations of polycyclic aromatic hydrocarbons (PAHs), also known as polynuclear aromatic hydrocarbons or polyaromatic hydrocarbons. PAHs consist of fused aromatic rings. Naphthalene is the simplest PAH with only 2 benzene rings, whereas all other PAHs have 3 or more fused rings. Some PAHs that have been identified in No. 6 fuel oil are phenanthrene, fluoranthene, pyrene, chrysene and benzo[a] pyrene (BaP).

1.2. Oil spill

In as much as oil is the main source of energy for the global economy, oil is sought and transported throughout the world but these activities lead to oil spills. An oil spill has been

defined as a discrete event in which oil is discharged through neglect, by accident, or with intent

over a relatively short time (Etkin, 2001).

1.2.1. History of oil spills

The history of oil spills is often thought to begin with the March 1727 sinking of the ship,

Torrey Canyon (Burgess, 1997). Although spills occurred earlier, the volumes were relatively

small. The Torrey Canyon was a supertanker and released 35 million gallons of oil. The accident

received wide media coverage and left a lasting public impression. The use of supertankers grew

and more spills happened. One of the more notable ones was the Exxon Valdez in 1989

(Peterson et al., 2003). Perhaps the most famous oil spill was a result of drilling at sea. This was

the blowout of Deep water Horizon (DWH) well in the Gulf of Mexico in 2010 (Peterson et al.,

2012). Usually oil spills have involved crude oil in the marine environment. However spills of

other oil types and in other environments also have occurred. An example of one was the Lake

Wabamun Incident.

1.2.2. The Lake Wabamun incident

Lake Wabamun is 19.2 kilometers long and 6.6 kilometers wide with a mean depth of 6.3 m

and lies 65 km west of Edmonton, Alberta, Canada. Having excellent fishing and being close to a

large urban centre, Lake Wabamun is considered as one of the most important recreational lakes

in Alberta. On Aug 3 2005, a CNR train derailed along the north shore of the lake, spilling

149,500 L of Bunker C and 46,850 L of pole treating oil into and around the lake. Although train

traffic was quickly restored, local residents felt the response to the oil spill was slow and

protested by temporarily blocking rail traffic. The accident and the subsequent actions generated

considerable attention from the media, which was often referred to it as the Lake Wabamun

incident (Alberta Environment, 2006)

1.2.3. Oil spill- treating agents

An assortment of agents has been developed to help in clearing up or removing oil from spill sites (Burger, 1997). Two similar related agents are dispersants and surface-washing agents. Both contain surfactants, chemicals like those in soaps and detergents. Surfactants reduce the oil-water interfacial tension and promote the coagulation of oil into droplets. However dispersants and surface-washing agents are used for different purposes. Dispersants promote the formation of small droplets of oil that disperse throughout the top layer of the water column. The effectiveness of dispersants is defined as the amount of oil put into the water column compared to the amount of oil remaining on the surface. By contrast surface-washing agents are applied to shorelines or structures to release oil from surfaces, with up to 95 % of the oil being released in some situations (Burger, 1997). The surface-washing agents are sprayed onto the shoreline and then sometime later they are washed off with a low-pressure water stream. Oil is retained in nearby water by the use of booms and removed by skimmers. Dispersants have been used much more extensively than surface-washing agents.

Corexit is one of the most famous oil spill dispersants. Corexit 9500 and 9527 are the most commonly used formulations and are produced by Nalco. After the DWH blowout on April 24, 2010, the primary dispersant applied in the Gulf of Mexico over the spring and summer of 2010 was Corexit. Approximately 8,000,000 liters was used, mainly Corexit 9500 because 9527 was deemed too toxic (Kujawinski et al., 2011; Major et al., 2012). In general terms, the ingredients include anionic surfactants, nonionic surfactants, and organic solvents. The organic solvents act to dissolve and suspend materials. One of the anionic surfactants is dioctyl sodium sulfosuccinate (DOSS). Other components include sorbian derivatives, 2-butoxyethanol, propanol, 1-(2-butoxy-

1-methylethoxy), and organic sulfonic acid salts (Major et al., 2012; Place et al. 2010; Singer et al., 1972; Wooten et al., 2012).

1.3. Toxicology/Ecotoxicology of oil spill

Oil spills are harmful to all aquatic animals (Burger, 1997). The nature of the impact and the mechanisms of toxicity depend on the exposure routes, oil types, and animal groups. Exposure routes can be through physical contact, ingestion, and inhalation of volatile components. The oil can range from jet fuel to crude oil, and the oil can be fresh or weathered. The animals include invertebrates, especially on the shoreline, fish, birds, and mammals (Kingston, 2002). Different toxicological mechanisms are possible and for any one incident multiple mechanisms are likely acting. Two very general ways that oil can act toxicologically is through physical contact and as a source of chemical toxicants. The chemicals include components of oil such as PAHs as well oil-spill treatment agents. These chemicals can act by general mechanisms, such as narcosis, or through specific cellular mechanisms, such as metabolism and activation of cell signaling pathways. These cellular mechanisms are particularly important for PAHs and can act singly or together to bring about toxicological consequences at the organismal level and ultimately population level. An example of this is blue sacs disease in fish. Some of these topics are expanded upon in the following sections.

1.3.1. Physical contact

Physical contact is one general way oil spills can be harmful to aquatic animals. Physical contact is especially detrimental for organisms that spend some or some part of their life cycle on the water surface or shoreline. Oil can physically smother shore invertebrates (Suchanek, 1993). Birds are killed when their feathers become covered in oil (Iverson & Esler, 2010). Feathers contribute to the insulation, waterproofing, buoyancy, and aerodynamic lift of birds. Normally

feathers exclude water but oil passes through feathers, causing them to become matted. This impairs or destroys all the functions of feathers. As a result, birds die of exposure and/or starvation. Physical contact with oil also kills aquatic fur-bearing mammals, such as sea otters. In this case the fur becomes matted and no longer provides insulation. The mammals ultimately die from the cold. For fish, physical contact is generally less important as a toxic mechanism because they can swim from areas with oil.

1.3.2. Narcosis

In narcrosis, the hydrocarbons of oil partition in the lipid bilayers of cellular membranes and disturb the organization and functions of membranes, which lead to the death of cells and organisms. This is a nonspecific mechanism and is sometimes referred to as baseline toxicity (Bradbury et al., 1989; McGrath et al., 2005). The narcotic efficiency correlates with the coefficient of partition between oil and water. The three ringed PAHs are thought to cause a low level of baseline toxicity attributed to non-polar narcosis (Mu et al., 2012, Schirmer et al., 1998; Van Wesel & Opperhuizen, 1995). Narcosis does not explain sublethal or long-term effects.

1.3.3. PAH metabolism

Organisms, including animals, metabolize some of the compounds in oil, including many PAHs (Schlenk et al., 2008). These biotransformations are part of xenobiotic metabolism and are done in two phases that broadly speaking makes nonpolar lipophilic chemicals into polar water-soluble metabolites. The metabolites can then be eliminated. Thus biotransformations can be considered as part of a detoxification process. However, some metabolites can be more hazardous than the parent compounds. Thus biotransformations can also be considered as bioactivation and the products can act as toxicants. The toxicants include mutagens and carcinogens. The enzymatic reactions of biotransformations are done in two phases. In the phase

I process, polar atoms are either added to the xenobiotics or exposed. Oxidation, reduction, and hydrolysis are the three general phase I reactions. In phase II reactions, the metabolites are acted upon to be either more polar or less bioactive.

The oxidative processes in phase I biotransformation are done primarily by the cytochrome P450 monooxygenase system (Schlenk et al., 2008). The cytochrome P450s (CYPs) make up a superfamily of heme-containing proteins and catalyze biological oxidations and reductions. CYPs catalyze the conversion of many PAHs into more toxic intermediates. The CYPs can be divided into constitutive and inducible forms. Constitutive CYPs appeared early in evolution are involved in anabolism (biosynthesis) of endogenous substances, such as steroids. Inducible CYPs (CYP1 through CYP4) came about later in evolution and mediate the catabolism (breakdown) of endogenous compounds (endobiotics) and xenobiotics.

CYP1A is likely the most studied inducible CYP form. In fish, there is some uncertainty as to whether multiple CYP1A genes exist and what nomenclature should be used to describe those (Schlenk et al., 2008). Therefore in this document both the gene and the protein will be referred to as CYP1A. In rainbow trout the protein is about 59 kDa. Some scientists use P450 1A to indicate the protein and reserve CYP1A for only the gene. In both mammals and fish, CYP1A can be measured as 7-ethoxyresorufin-o-deethylase activity (EROD). In both mammals and fish, CYP1A is responsible for the metabolism of the PAH, benzo[a] pyrene (BaP) (Schlenk et al., 2008; Schirmer et al., 2000).

1.3.4. CYP1A induction as a biomarker

Many of the substrates for CYP1A are also inducers. For example, in fish BaP is both an inducer and a metabolite (Schlenk et al., 2008; Bols et al., 1999). Therefore many inducers stimulate their own metabolism. Early studies on the effect of crude oil on brown trout noted that

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CYP1A-mediated activities were elevated (Payne & Penrose, 1975). Therefore, elevated EROD activity or other measures of P450 1A became a biomarker of exposure to oil pollution (Whyte et al., 2000; Wiedmer et al., 1972). In fact some have argued that CYP1A induction is a bioindicator (Carls et al., 2005). Induction in early life stages indicates long-term negative consequences for individuals and populations. The induction mechanism for CYP1A involves the aryl hydrocarbon receptor (AhR) signaling pathway.

1.3.5. Activation of the aryl hydrocarbon receptor (AhR)

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. Historically the AhR is known for regulating responses to an array of environmental chemicals (Hahn et al., 2009; Furness & Whelan, 2009). This includes some PAHs but the most studied ligand has been 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The pathway by which TCDD acts at the cellular level is well delineated (Figure 1.2). Upon binding TCDD, the AhR translocates to the nucleus, forms a transcriptional activator that binds to specific DNA enhancer sequences (dioxin response elements), and regulates gene expression. The ligands that activate the AhR are sometimes known as dioxin-like compounds (DLCs). Although this could include some PAHs, usually the use of the term DLCs has been restricted to just the members of the dioxins, furans and polychlorinated biphenyls (PCBs) that act through AhR. The best understood target gene for AhR is CYP1A. However, increasingly AhR is being found to have roles in normal animal physiology and development, independent of xenobiotic ligands. These include involvement in regulating cell migration, growth, death and roles in vascular development and functioning of the immune system. The endogenous ligands have yet to be conclusively identified but some possibilities are tetrapyrroles, indoles and arachidonic acid metabolites (Furness & Whelan, 2009).
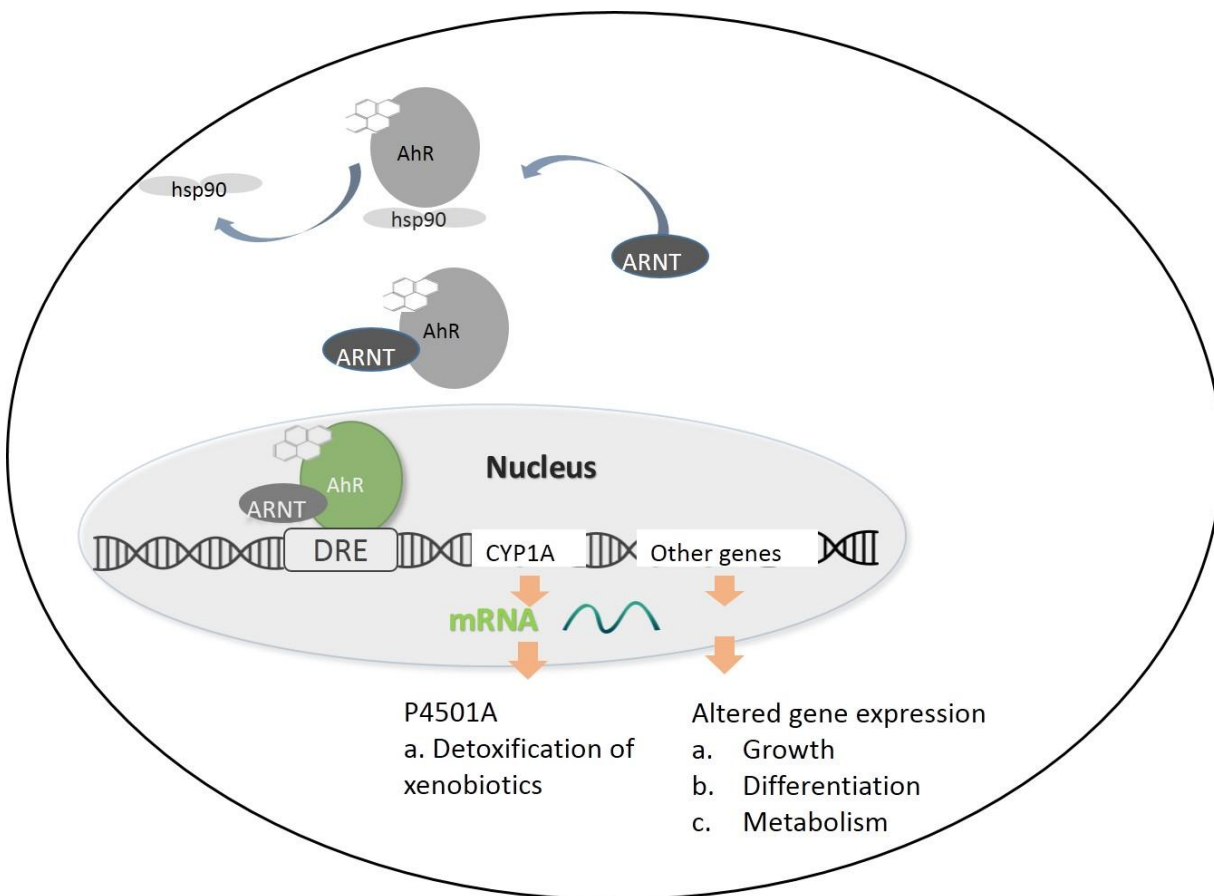
Figure 1.2.The molecular mechanism of Cytochrome P450 induction though Aryl hydrocarbon Receptor (AhR) Pathway*. Ligands such as PAHs binds with the Ah receptor, which is located on the inner layer of cell membrane. The AhR complex is then translocated into nucleus and bind with the promoter region of CYP1A genes or alternative genes. The binding between AhR-ligand complex and DNA initiates the transcription and translation of various Phase I and II enzymes including CYP1A.*

1.3.6. Blue Sac disease in fish

Blue sac disease (BSD) involves abnormalities in the early development of fish (Wolf, 1957). Experimentally, exposing fertilized fish eggs to dioxin-like compounds (DLCs) causes the features of blue sac disease (Walker et al., 1972). Some of these are spinal deformities, craniofacial malformations, hemorrhaging, yolk and pericardial edema, impaired cardiac development, and circulatory failure (Hornung et al., 1999; Marty et al., 1997). These symptoms lead to growth inhibition and mortality. Exposure of fertilized eggs from several fish species to PAHs (Barron et al., 2004; Billiard et al., 1999) or crude oil (Carls et al., 1999; McIntosh et al., 2010) also causes BSD. By reducing the recruitment of young into the adult fish population BSD could be one route by which oil spills impact local fish populations.

How dioxin-like compounds (DLCs) and PAHs cause early life stage abnormalities is still a matter of debate. Most of the debate focuses on the involvement of CYP1A and the AhR signaling pathway. Initially DLCs and PAHs were both thought to exert their embryotoxicity through a similar mechanism in which CYP1A induction via the AhR pathway was crucial. However more recent research suggests that multiple pathways could be involved in embryotoxicity and the mechanisms for DLCs and PAHs could differ (Incardona et al., 2005; Mu et al., 2012; Scott et al., 2011). Despite the correlation of CYP1A induction by oil with early life stage abnormalities (Carls et al., 2005), cardiac dysfunction by three ringed PAHs was not due to narcosis and more importantly independent of the AhR (Incardona et al., 2004, 2005). The PAH, retene, appeared to act through an isoform of AhR (AhR2) to cause cardiovascular toxicity in zebrafish embryos (Scott et al., 2011). However the toxicity was independent of CYP1A induction. Indeed, several studies have examined the effect of inhibiting P450 1A (CYP1A) activities on the embryotoxicity of PAHs (Fleming & DeGuilo, 2011; Hodson et al., 2007; Mu et

al., 2012). Both synergism and antagonism have been observed. Overall the results suggest that multiple mechanisms are involved in the impairment of early life stage by PAHs and oil but any CYP1A and AhR involvement might vary with the composition of the oil or with different PAHs.

1.4. Use of animal cell cultures in oil spill research

Animal cell cultures offer several advantages as experimental tools to study the toxicity of oil. Firstly, studies with cell cultures satisfy a societal desire to reduce the number of animals that are used in toxicology testing. Secondly, cellular phenomena can be studied in a controlled and, in some cases, a completely defined environment, without the confounding variables of systemic or larger physiological controls. Oil is a complex mixture, and attempts to find the toxic compounds or fractions in the mixture are more easily done with animal cell cultures than with animals. Relative to exposing animals, dosing of cell cultures is easier, more reproducible, requires less toxicant, and produces less toxic waste. Results are obtained more rapidly and the cost of the test is less than with whole animals.

Animal cell cultures have been used intensively to study individual PAHs but the in vitro approach has been applied much less often to the toxicology of oils or oil fractions. For the priority PAHs, their cytotoxicity and photocytotoxicity has been studied with the rainbow trout gill cell line, RTgill-W1 (Schirmer et al., 1998a&b) and their ability to induce EROD activity has been determined with rainbow trout liver cell line, RTL-W1 (Bols et al., 1999). Among oil products, jet fuel has been studied intensively (Chou et al., 2003; Espinoza et al. 2003; Grant et al., 2000; Ramos et al., 2007). Recently crude oil has been examined in the rat hepatoma cell line, H4IIE, for both AhR and estrogenic receptor activation (Vrabie et al., 2009, 2011). However, few studies have focused exclusively on heavy fuel oil (HFO).

1.5. Research on the Lake Wabamun incident

Obvious environmental effects were seen immediately and for some time after the Lake Wabamun spill (Goodman, 2007). High wind spread oil across a significant part of the lake surface. Later oil washed up on most of the north, east and southeast shoreline. Birds and other wildlife were found covered in oil and efforts were made by volunteers to rehabilitate them. Dead fish were found. Tar balls and other aggregates of oil formed and were still being seen two years later.

Environmental assessments done in the first few years of the spill have suggested that the spill has not seriously impacted Lake Wabamun. However, one concern has been the long-term impact on fish populations. Oil was detected in the bottom of the lake and could be a chronic source of PAHs. These could negatively impact early fish life stages and reduce the recruitment of young into fish populations. In order to lay the groundwork for understanding this risk, efforts have been recently undertaken to fractionate the Bunker C (heavy fuel oil 7102) from the Lake Walbamun spill and to determine the effects of different fractions on fish embryos.

In order to identify the toxic components and assess their toxicity, a four-phase chemical analysis of Bunker C was performed (Bornstein, 2012). The fractionation scheme is illustrated in Figure 1.3. The oil was separated into three fractions based on the boiling temperature of n-alkanes with different carbon numbers by low-temperature vacuum distillation. One of these fractions, F3, was subject to a series of solvent extractions to remove asphaltenes and waxes. Open column chromatography was used to further fractionate F3. F3-1 was rich in PAHs; F3-2, in waxes. The chemical composition and the distribution of PAHs and alkylated PAHs in the whole oil and its fractions were analyzed by GC-MS and HPLC and are listed in Appendix A. Enough material for toxicity testing was available for only some of the factions.
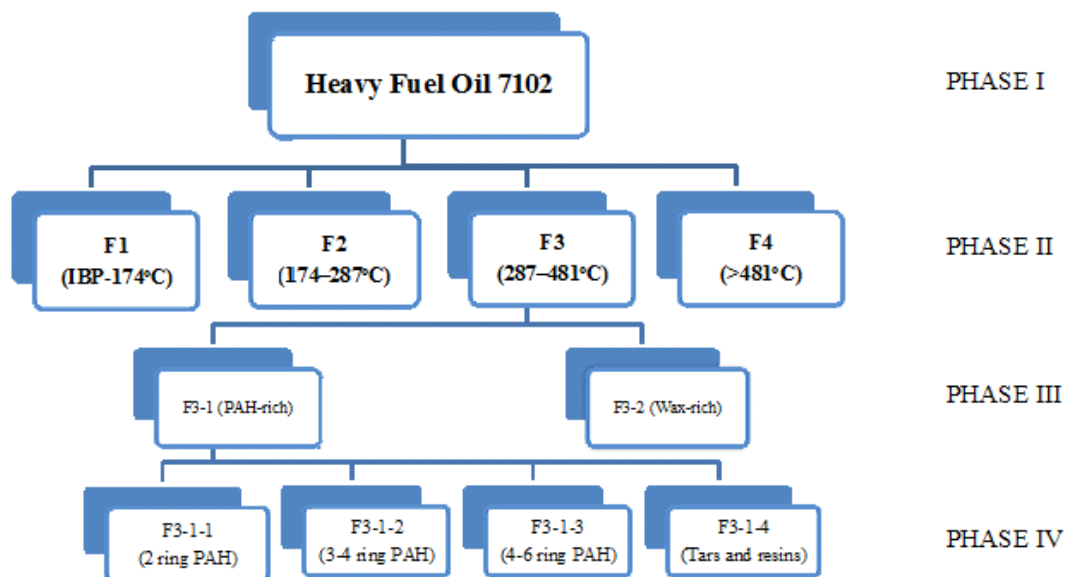
Figure 1.3. Effects-Driven Fractionation Flow Chart for HFO 7102. *The chemical analysis was performed by Jason Bornstein at Queen's University. Phase II separation was based on the boiling temperature of n-alkanes with different carbon numbers. Three fractions, F2, F3 and F4 were generated from low temperature vacuum distillation. Fraction 3 was further separated by modified cold-acetone extractions in Phase III. Two more fractions were generated: F3-1 (PAH-rich extract) and F3-2 (wax-rich residue).*

Embryo toxicity tests have been done by Martin (2011) and Bornstein (2012) as part of their MSc theses and continue to be done by Julie Adams, as part of her MSc program at the Queen's University, Kingston, Ontario. For the toxicology studies, HFO 7102 and its fractions were tested as Water Accommodated Fraction (WAF) or Chemically Enhanced Water Accommodated Fraction (CEWAF). WAF contains dissolved oil components and microdroplets. CEWAF is made by the same method as WAF but at the end the addition of chemical dispersant Corexit 9500 is made.

1.6. Overarching objective and specific objectives

The overarching objective of this thesis is to study the toxicity of HFO 7102 and its fractions to the rainbow trout liver cell line, RTL-W1. The fractions are those developed by Bornstein (2012) and are fractions F2, F3, F3-1, F3-2, and F4. They will be tested primarily as CEWAF. Ultimately the results that are obtained with the fish cell lines will be compared with the embryo tests that have been done and are being done at Queen's University. The specific objectives for the in vitro approach of this thesis are outlined below in point form:

A. Determine the cytotoxicity of Corexit 9500 to rainbow trout cell line, RTL-W1.

B. Determine to the cytotoxicity of HFO 7102 and its fractions, F2, F3, F3-1, F3-2 and F4 HE-CEWAFs to rainbow trout cell line, RTL-W1

C. Assess the ability of Corexit 9500 to induce CYP1A expression and activity in rainbow trout cell line, RTL-W1

D. Assess the ability of HFO 7102 and its fractions, F2, F3, F3-1, F3-2 and F4 HE-CEWAFs to induce CYP1A expression and activity in rainbow trout cell line, RTL-W1

E. Evaluate the effects of long term exposure to F3 on the CYP1A expression and activity in

    RTL-W1

Chapter 2 Cytotoxicity to RTL-W1 of Corexit, HFO 7102 and its fractions HE-CEWAFs

2.1. Introduction

PAHs are often found in tissues of fish from areas that have experienced oil spills. PAHs generally have high octanol-water partition coefficients (Kow), which keep them from dissolving into static water column. However, bioavailability of PAHs in spilled oil could be increased by high-energy turbulence or the application of chemical dispersants, such as Corexit 9500 (Schein et al., 2008; Ramachandran et al., 2004). More than 100 PAHs have been identified in the environment but only a few of their toxic mechanisms were well documented. 16 PAHs are considered as priority PAHs by US government and become the main targets for measurement of environmental pollutants due to their carcinogenicity, mutagenicity and/or teratogenicity (ATSDR, 1995).

2.1.1. Corexit 9500 and its cytotoxicity studies

The low-solubility of oils in water makes them difficult to study on organisms, including studies being done in vitro. A dispersant, as a surface-active reagent, largely reduces the water-oil surface tension (Place et al., 2010). In the case of the Deepwater Horizon oil spill in the Gulf of Mexico, Corexit 9500 was used to break the crude oil emulsion into small globules (Kujawinski et al., 2011). Corexit 9500 is a commercial oil dispersant manufactured by Naclo Holding Company. The major components of Corexit 9500 are propylene glycol, petroleum distillates hydrotreated light fraction, sorbitan oleate emulsifying agents and proprietary organic sulfonate, which disclosed to EPA as dioctyl sodium sulfosuccinate (DOSS; Naclo, 2010; Benner Jr et al., 2010). Even though the risk of using Corexit was assessed by US EPA, it is not yet fully

understood exactly how Corexit interacts with organelles and proteins in order to elicit adverse effects on aquatic organisms.

2.1.2. Cellular mechanisms of PAHs cytotoxicity

In the past few decades, the cytotoxicity of either single PAHs or PAH mixtures were intensively assessed in several human and animal cell lines (Babich et al., 1988; Godard et al., 2006; Schirmer et al., 1998; Tarantini et al., 2011; Vrabie et al., 2009). Some representative PAHs as environmental pollutants include benzo[a]pyrene (BaP), benzo[k]fluroanthene (BkF), benzo[b]fluorene (BbF), pyrene, chrysene, fluoranthene (Fl), naphthobenzothiophene (NBT) and dibenzothiophene (DBT; Mumtaz et al., 1972; Pickering & Phil, 2000).

Different PAHs exert toxicity through various cellular mechanisms. Small PAHs with two or three benzene rings could be cytotoxic directly. Some PAHs could be activated by UV irradiation and damage cells through formation of singlet oxygen and free radicals (Choi & Oris, 2003; Schirmer et al., 1998; Wang et al., 2007; Zhang et al., 2006); while other PAHs could be activated enzymatically. The most common process is through inducing cytochrome P450 superfamily (CYP), which is a group of enzymes involved in catalysis of oxidation of organic compounds. For example, menadione and its *o*-quinones metabolites generated by dihydrodiol dehydrogenase were reported to be cytotoxic in H4IIE cells. The impaired cell viability and/or cell survival were due to the production of superoxide anion radicals, *o*-quinones anions or alternative radicals (Flowers-geary et al., 1996). In addition, some PAHs such as BaP were reported as having the ability to activate endocrine signaling pathways (Willing et al., 2011). When cells are exposed to PAH mixtures, multiple processes could be triggered and elicit lethal effects together.

2.1.3. Monitoring Cell viability

In general, both Corexit and heavy fuel oil (HFO) are a mixture of chemical compounds. When the effective concentrations are reached, cellular stresses were generated from the activation of many pathways, which lead to organelles damage. Thus, to monitor the cytotoxicity of Corexit or HFO and its fractions, two florescent dyes, Alamar Blue and CFDA-AM, were employed to assess the cytotoxicity of HFO 7102 and its fractions HE-CEWAFs. Alamar Blue is a fluorescent dye, which freely diffuses into living cells. It can be reduced to resorufin by reductase located in mitochondrial membranes as well as in the cytosol. Reduction causes the dye to fluoresce at unique excitation and emission wavelengths. Integral mitochondrial membranes are required to maintain the normal function of electron transport chain (Schreer et al., 2005). 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) can diffuse into cells and be converted into fluorescent product by nonspecific esterases. The CFDA- AM assay needs the plasma membrane remain intact in order to maintain a cytoplasmic milieu that will support esterase activity and the formation of a fluorescent product (Schirmer et al., 1997). A decline in fluorescent readings from both dyes indicates impaired cell viability.

2.1.4. Objectives

The objectives of this chapter are to evaluate the cytotoxicity of Corexit 9500 and HFO 7102 and its fractions through the use of the rainbow trout liver cell line, RTL-W1.

2.2. Methods & Materials

2.2.1. Maintaining and culturing RTL-W1

The rainbow trout liver cell line (RTL-W1) was established by Lee *et al*. (1993). RTL-W1 cells were maintained in 75-cm^2 culture flasks at room temperature with Leibovitz's L-15 culture medium (HyClone Laboratories, Inc., Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin- streptomycin solution (10,000 units/ml penicillin, 10 mg/ml streptomycin, HyClone).The subcultivation procedures have been previously described (Bols et al., 1994; Schirmer et al., 1994), except TrypLE (Life Technologies) rather than trypsin was used to remove cells from the plastic growth surface.

2.2.2. Preparation of Corexit 9500

A Corexit stock solution (0.1 % v/v) was prepared by mixing 1 μl Corexit (Nalco Energy Services, Sugar Land, TX) with 10 ml of three different media: L-15 supplemented with 10% FBS, L-15 medium, or L-15/ex respectively. L-15/ex is a modification of L-15, which contains salts, galactose and pyruvate without vitamins or amino acids. The preparation procedure for L-15/ex were as outlined in Schirmer et al. (1997). Stock solutions were serially diluted in their respective media and the concentrations were expressed as % (v/v).

2.2.3. Preparation and dilution of HE-CEWAFs of HFO 7102 and its fractions

To simulate the dissolution of hydrocarbons from spilled oil by wind and wave action and dispersion facilitated by addition of chemical dispersant, HE-CEWAF of each oil sample was used to expose RTL-W1. HE-CEWAF preparation protocol was modified from Singer et al. (2000) and was provided by Julie Adams from Queen's University. HFO 7102 (Bunker C) was fractionated into 5 fractions (F2, F3, F3-1, F3-2, and F4) at Dr. Brown's laboratory (Queen's University) by Jason Bornstein (2012).

The scheme for preparing HE-CEWAF is shown in Appendix B Figure B.1. Briefly, around 100 μl of heavy fuel oil or its fractions were weighed out and added into a 20 ml silicon glass sample vial which contained 10 ml of cell tissue culture grade water, in order to achieve an oil to water ratio of 1:100. 10μl of Corexit 9500 was then applied onto the oil layer, which gave a dispersant to oil ratio of 1:10. By establishing the oil emulsion, oil-water mixture was mixing for 5 minutes by Vortex Genie 2, under its maximum speed. By settling on lab bench for 1 hour, the oil emulsion was separated into two layers: large oil droplets on the top oil phase and oil or its fractions HE-CEWAF stock at the bottom aqueous phase. Oil/fraction HE-CWAF stock solution was removed from sample vial and was serially diluted into L-15 medium. The concentration of HE-CEWAF is expressed as mg/ml and the final Corexit concentration in HE-CEWAF stock solution was 0.1 % (v/v). To control the number of variables, the dilution factors for all the HE-CEWAF samples are the same. Key parameters and dilution factors are listed in Appendix B Table B.1 . Due to the volatility and low water solubility of PAH, both HE-CEWAF stock solutions and the dilutions were made just before dosing cells.

2.2.4. Exposure of RTL-W1 to Corexit 9500 or HE-CEWAFs of HFO 7102 and its fractions

Confluent monolayers of RTL-W1 cells in 96-well tissue culture plates were used to evaluate cytotoxicity. Confluency was achieved by plating 50,000 cells per well and allowing them to grow for 3 days.

One 96 well plate of RTL-W1 were used to evaluate each test solution. After 3 days, culture media were discarded and each well was rinsed with 50 μl Phosphate-buffered saline (PBS, BioWhittaker). Wells then received 200 μl of Corexit or of diluted HE-CEWAF. Each concentration was repeated in 6 wells. To terminate the exposures, the media were removed from microplates and each well was rinsed by 50 μl PBS.

2.2.5. Cytotoxicity Assays

At the end of exposure periods, the morphology of RTL-W1 was assessed by examining cultures with an inverted phase contrast microscope and the cell viability was measured with two fluorescent indicator dyes: Alamar Blue for general cellular metabolic activity; 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) for plasma membrane integrity. Alamar Blue and CFDA-AM was purchased from Molecular Probes, Engene, OR, USA. Detailed protocols for using these dyes have been published and these were by in large followed (Dayeh et al., 2003; Schirmer et al., 1997). Key steps of measuring cytotoxicity by Alamar Blue and CFDA-AM was shown in Appendix C Figure C.1. Working solutions of Alamar Blue, CFDA-AM were applied into each well with 1 hour incubation in dark. At endpoint, fluorescence was quantified with CytoFluor 4000 (PerSeptive Biosystems, Burlington, ON, Canada). Excitation and emission wavelength for Alamar Blue were 530 nm and 595 nm, 485 nm and 530 nm for CFDA-AM. After 1 h with the indicator dyes, fluorescence was quantified with a fluorometric plate reader, CytoFluor 4000. The results were recorded as relative fluorescence units (RFUs).

2.2.6. Analyzing data

The RFUs for treated culture wells were expressed as a percentage of the RFUs in control culture wells. Raw data was first analyzed using Microsoft Excel. Background RFUs from blank wells were first subtracted. RFUs from treated wells were averaged and then expressed as a percentage of the average fluorescent readings of the control wells. Each data points was transformed into log scale and were analyzed by non-linear regression with a sigmoid relationship using the curve-fitting route. The EC_{50}s are the concentrations that produce 50% viability. It were calculated using a four-parameter logistic equation. To test the significant differences between results, one-way ANOVA with Tukey's test ($\alpha \leq 0.05$) was conducted. All

graphs, statistical analyses and EC$_{50}$ values were calculated using GraphPad Prism version 4.00 (GraphPad Software, San Diego, USA).

2.3. Results

The cytotoxicity of HFO 7102 and its fractions to RTL-W1 was screened through the use of two fluorescent indicator dyes, Alamar Blue and CFDA-AM. Due to the hydrophobicity of oil, Corexit 9500 was used to facilitate the dissolution of oil and its fractions into cell culture medium. To eliminate the possible toxicity introduced by the use of Corexit, the cytotoxicity of Corexit 9500 on RTL-W1 was first tested in three different types of media: L-15/ex, L-15 and L-15 with 10 % FBS.

2.3.1. Cytotoxicity of Corexit 9500

Both mitochondrial activity (screened by Alamar Blue) and cell membrane integrity (screened by AFDA-AM) of RTL-W1 were impaired by Corexit 9500 in three different cell culture media (Figure 2.1). However, Corexit diluted in different cell culture media elicited cytotoxicity in different concentrations. From dose-response curves established based on the screen results from both dyes (Alamar Blue and CFDA-AM), Corexit in L15/ex were clearly more potent than it diluted in other two cell culture media (L-15 or L-15 supplemented with 10% FBS).

To further compare their potencies, EC_{50} for each treatment was estimated as described in section 2.2.4 and listed in Table 2.1. The potency of Corexit in three cell culture media determined by Alamar Blue and CFDA-AM are similar. By Alamar Blue: L-15/ex (0.0023 %; n=1) > L-15(0.0117 ±0.0059 %; n=3) > L15 with 10% FBS (0.1023±0.0178 %; n=3); By CFDA-AM: L-15/ex (0.0038 %; n=1) > L-15 (0.0125±0.0050 %; n=3) >L-15 with 10% FBS (0.1021 ±0.0194 %; n=3). The differences between L-15 and L-15 with FBS were tested by t test (p<0.05).
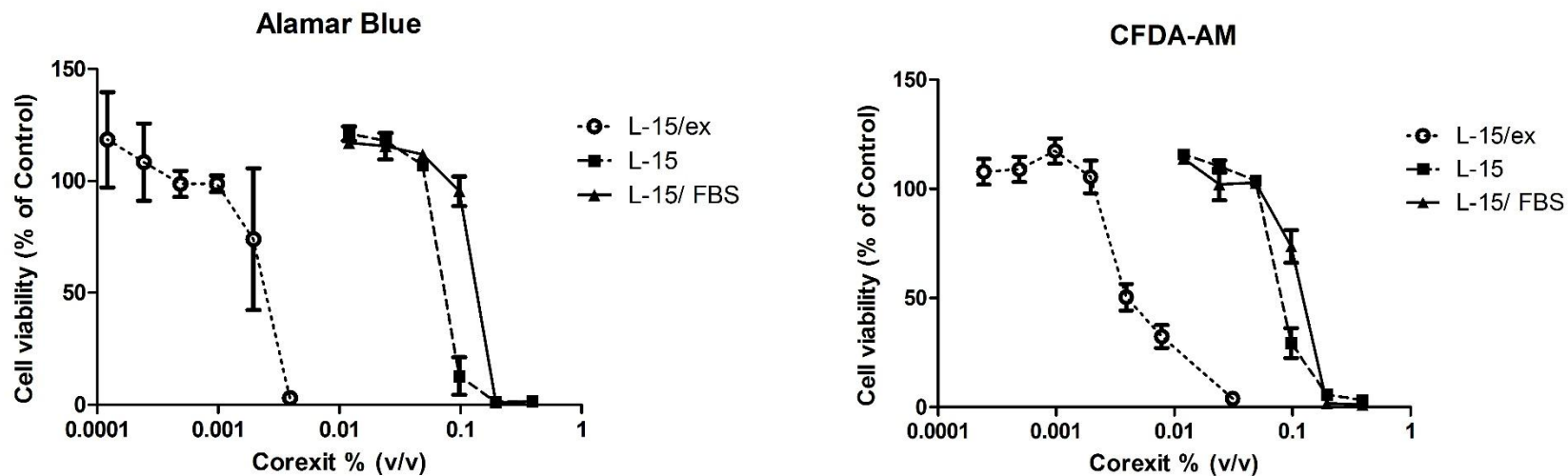
Figure 2.1.Impaired viability of RTL-W1 exposed to increasing concentration of Corexit 9500 in different culture media. *Confluent cultures were exposed to Corexit 9500 in different media: L-15/ex (○), L-15 (■) and L-15 supplemented with 10% FBS (▲) for 24 hours. At the endpoint, Corexit dilutions were removed and cell viability of each well was measured with two fluorescent indicators: Alamar Blue, CFDA-AM. Toxic effects on cells were expressed as the percentage of the fluorescent reading of control. One representive experiment is shown. Each data point represents the mean of six culture wells with the standard deviation illustrated as vertical bar.*

Table 2.1. Cytotoxicity of Corexit diluted in L-15 with 10% FBS, L-15 and L-15/ex in RTL-W1

Corexit in different media[1]	EC50s[3] (% v/v)± SD (n)	
	Alamar Blue	**CFDA-AM**
L-15/ex[2]	0.0023 (1)	0.0038 (1)
L-15	0.0117[a] ±0.0059 (3)	0.0125[b] ±0.0050 (3)
L-15/FBS	0.1023[a] ± 0.0178 (3)	0.1012[b] ±0.0194 (3)

[1]*Corexit 9500 was diluted in different cell culture media: L-15/ex, L-15 and L-15 supplemented with 10% FBS*
[2]*L-15/ex is a modification of L-15, which contains salts, galactose and pyruvate without vitamins or amino acids*
[3]*Except for L-15/ex, each EC 50 showed in the table are the average from three trials; the concentration of Corexit was expressed as the percentage of Corexit volume in the total media volume*
[a,b] *means within each column with the same letter were statistically different with each other as judged by t test ($p \leq 0.05$)*

2.3.2. Cytotoxicity of HFO 7102 and its fractions HE-CEWAFs

After 24 h exposures HFO 7102 and some of its fractions were cytotoxic to RTL-W1 at the highest testable concentrations. Representative curves are shown in Figure 2.2. The three PAH rich fractions, F2, F3 and F3-1, showed cytotoxicity. By contrast, the asphaletane fraction, F4, and the wax-rich fraction, F3-2, were not cytotoxic up to the concentration of 1 mg/ml and 20 mg/ml, respectively. To rank these samples, EC_{50} values of HFO and its three PAH-rich fractions were estimated from corresponding dose-response curve (Table 2.2).

The potency of HFO 7102 and its fractions HE-CEWAFs determined by Alamar Blue is: F3 (0.478 ± 0.077 mg/ml; n=3) > F2 (0.891 ± 0.034 mg/ml; n=3) ≈ F3-1 (0.721 ± 0.186 mg/ml; n=3) > HFO 7102 (2.723 ± 0.968 mg/ml; n=3); The potency determined by CFDA-AM is: F3 (0.410 ± 0.061 mg/ml; n=3) > F2 (0.902 ± 0.043 mg/ml; n=3) ≈ F3-1 (0.694 ± 0.163 mg/ml; n=3) > HFO (2.920 ± 0.868 mg/ml; n=3). For all samples, EC_{50} values obtained in Alamar Blue and CFDA-AM assay are similar. The differences between samples were analyzed by One-way ANOVA ($\alpha= 0.05$). Indicated by both dyes, the potency of HFO is significantly lower than its fractions; no differences were observed between fractions. However, this could be due to small sample size (n=3) and large standard deviation, which is proven by the none overlapping 95 % Confident Interval between F2 and F3, also F3 and F3-1. After excluding EC_{50} value of HFO, EC_{50} values between F2, F3 and F3-1 are different, determined by One-way ANOVA analysis followed by Turkey's post-test ($\alpha= 0.05$).
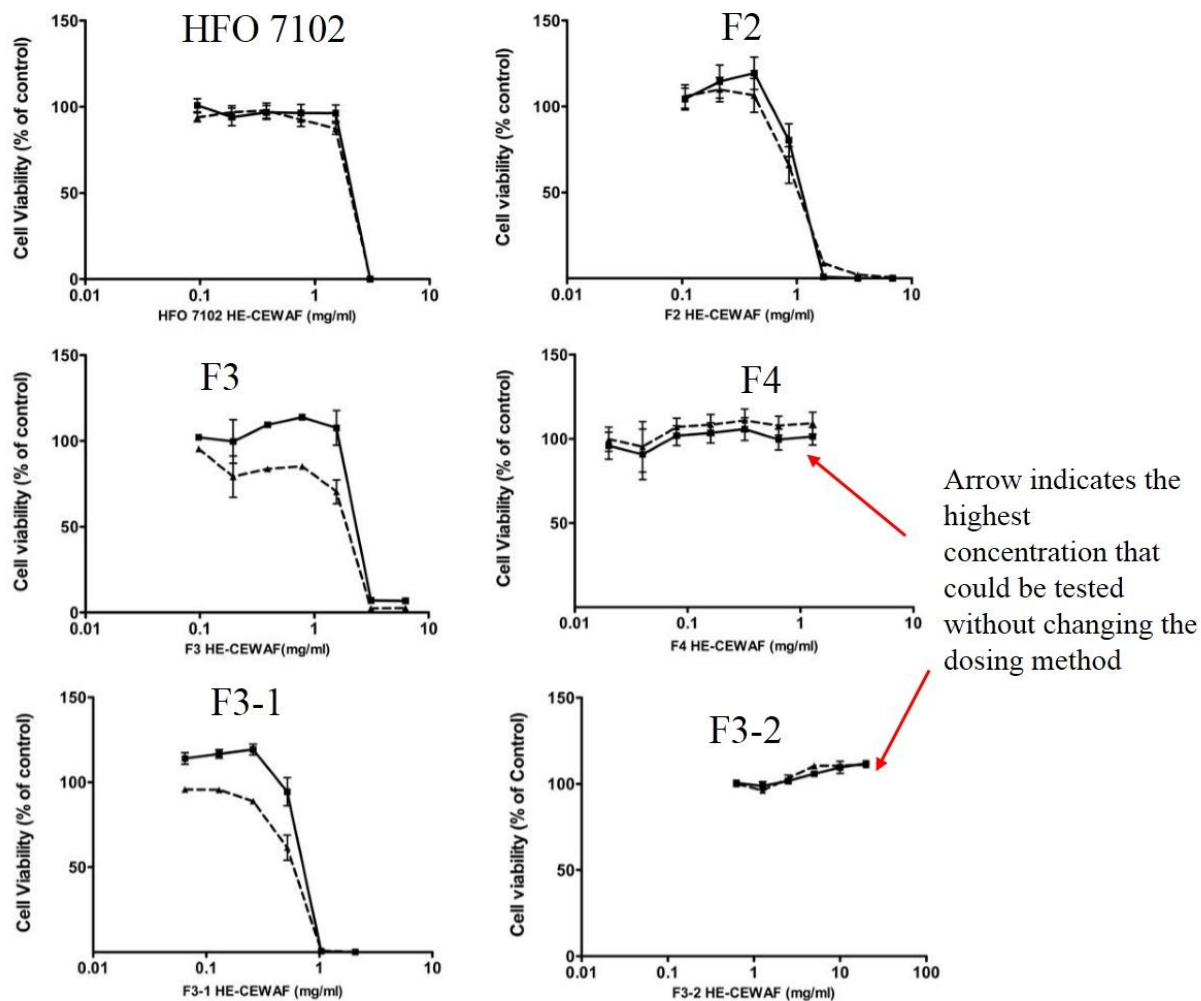
Figure 2.2. Impaired viability of RTL-W1 cells upon exposure to increasing concentrations of HFO 7102 or its 5 fractions HE-CEWAFs. Confluent cultures were exposed to HFO 7102 or its fractions HE-CEWAFs for 24 hours. At the endpoint, HE-CEWAFs in culture media were removed and cell viability of each well was measured with two fluorescent indicators: Alamar Blue (■), CFDA-AM (▲). Toxic effects on cells were expressed as the percentage of the fluorescent reading of control wells. One representive experiment is shown. Each data point represents the mean of six culture wells with the standard deviation illustrated as vertical bar.

Table 2.2. Cytotoxicity of HFO 7102 and its Fractions HE-CEWAFs in RTL-W1

Sample	Alamar Blue					CFDA-AM				
	EC_{50} (mg/ml)	SD	n	95 % Confidence interval		EC_{50} (mg/ml)	SD	n	95 % Confidence interval	
				lower 95% CI	high 95% CI				lower 95% CI	high 95% CI
HFO 7102*	2.723	0.968	3	0.318	5.128	2.920	0.868	3	0.764	5.076
F2	0.891	0.034	3	0.807	0.976	0.902	0.043	3	0.794	1.010
F3	0.478	0.077	3	0.236	0.618	0.410	0.061	3	0.257	0.562
F3-1	0.721	0.186	3	0.273	1.167	0.694	0.163	3	0.289	1.100

EC_{50} values in the table represent three replicate trials. Cytotoxicity of each sample was evaluated by Alamar Blue and CFDA-AM and expressed as cell viability percentage comparing with fluorescent reading in control wells
* By One-way ANOVA analysis, Mean EC_{50} value of HFO 7102 is significant different with the others ($p<0.05$)

2.4. Discussion

In order to determine non-lethal concentration of Corexit 9500 and the suitable culture condition for RTL-W1, Alamar Blue and CFDA-AM assay were used to evaluated the cytotoxicity of Corexit 9500 alone in three different media: L-15/ex, L-15 and L-15 supplemented with 10% FBS. Within none toxic Corexit concentration, HE-CEWAFs of HFO 7102 and each fractions was prepared and used to expose RTL-W1 in L-15 with FBS media. The cytotoxicity of HFO and its fractions to RTL-W1 were assessed by Alamar Blue and CFDA-AM as well.

2.4.1. Cytotoxicity of Corexit 9500 in L-15with 10% FBS, L-15 and L-15/ex

Cell viability of RTL-W1 treated with Corexit was monitored by two fluorescent indicators, Alamar Blue and CFDA-AM. Alamar Blue screens the metabolic activities in cell; CFDA-AM is an indicator for cell membrane integrity. Declined fluorescent readings from both dyes indicate impaired cell viability.

In the current study, the results from both indicators revealed that Corexit is cytotoxic to RTL-W1 in all three different cell culture media. Comparing their EC_{50} values, Corexit diluted in L-15/ex is most toxic, while Corexit diluted in L-15 or diluted in L-15with 10% FBS are about five fold and fifty fold less toxic than L-15/ex, respectively. At toxic concentrations, the decreased fluorescent reading from both dyes indicated that both cell membranes integrity and mitochondrial activities were impacted by Corexit (O'Connor et al., 1991; Goegan et al., 1995).

As a mixture, the underlying mechanisms of Corexit toxicity could be several. One possible reason could be the generation of reactive oxygen species (ROS). Studies reported that HepG2/C3A cells dosed with Corexit 9500 showed elevated oxidative stress and decreased

mitochondrial activity (Bandele et al., 2012). Specifically, the accumulation of reactive oxygen species is contributed by dioctyl sodium sulfosuccinate (DOSS), which is one of the main ingredients in Corexit 9500 (Bandele et al., 2012; Naclo Co, 2010). Other biological targets of Corexit were also screened in Judson et al studies. A battery of genes was turned on after exposed to Corexit. However, these results could be generally due to oxidative stresses rather than being specific targets of Corexit (Judson et al., 2010).

In addition, FBS and amino acids in L-15 could protect RTL-W1 though binding to ROS (Goegan et al., 1995). Reducing oxidative stress leads to less cytotoxicity and intracellular disruption. Since no toxic effects were observed under 0.05% Corexit in L-15with FBS, 0.1% of Corexit was chosen to make oil/fractions HE-CEWAFs stock solution. After further diluted the stock solution in L-15/FBS, Corexit concentration would be too low to exert toxic effects in RTL-W1.

2.4.2. Cytotoxicity of HFO 7102 and its fractions HE-CEWAFs

Comparing the cytotoxicity of HE-CEWAFs of HFO 7102 with its first three fractions obtained from Phase II distillation: F2, F3, and F4, F3 is the most cytotoxic, while F2 is intermediate, HFO 7102 is the least cytotoxic and F4 is not cytotoxic up to 1 mg/ml. The order of oil and its fractions potency is coincident with the levels of TPAHs in the whole oil and its fractions, which is F3 > F2 > HFO >F4 (Appendix A Table A.2). Generally, higher total PAH concentration in oil/fraction HE-CEWAF samples results in greater toxicity. F4 is not cytotoxic because it mainly composed of asphaltenes and resins, which are normally not cytotoxic due to their high molecular weight and low water solubility. The percentage of each component in the whole oil is: F2:F3:F4 =7%:25%:66% (Appendix A Table A.1). Therefore, HFO 7102, which

contains large percentages of resins and asphaltenes, is not as toxic as other light oils and petroleum products, since most of its toxic effects are contributed by PAHs.

Different PAHs composition in HFO and its fractions could affect the potency of oil and its fractions. As it was shown in Appendix A Table A.3, half of quantified PAHs in F2 are two rings and one third are three rings; half of the quantified PAHs in F3 are four rings and one third are three rings, the rest are mixture of 2, 5 and 6-ring PAHs. Even though 2-ring PAHs are more water soluble than PAHs with more rings (Plant et al., 1983), the solubility of 4, 5 and 6-ring PAHs could be sharply increased by the use of dispersant, in this case, the Corexit 9500. F3 HE-CEWAF, which is dominated by 4, 5, and 6 rings PAHs, was more cytotoxic than F2, which has more 2 and 3 ring PAHs. Possibly this is because besides direct cytotoxicity of the PAHs, some PAHs are being metabolized to cytotoxic products (Godard et al., 2006; Pickering & Phil, 2000).

However, the cytotoxicity of F3 is greater than F3-1, even F3-1 contains more PAHs. This could be resulted by different PAHs distribution in these two fractions. In phase III extraction, although F3-1 was extracted from F3, the PAH profiles of F3 and F3-1 are not identical. From the gas chromatography of F3 and F3-1 (Bornstein, 2012) F3-1 is concentrated with lighter PAHs. Therefore, the higher cytotoxicity observed in F3 is also resulted by the activation of CYP1A.

2.4.3. General Conclusions

Decreased cell viability is one of the signs that cells are experiencing stress. However, numerous pathways and processed triggered by different PAHs could be the causes. After the Gulf of Mexico oil spill, researches have been focusing on explore the toxicity of oil-dispersant mixture, instead of single PAHs. So far, ROS generations, free radicals, apoptosis, necrosis, and

autophagy have been reported in response to dispersants (Niestroy et al., 2011; Wang et al.,

2012).

Chapter 3 CYP1A induction in RTL-W1 by HFO 7102 and its Fractions as HE-CEWAFs

3.1. Introduction

CYP1A induction is a sensitive and specific adaptive response of organisms exposed to environmental pollution, including oil spills (Bucheli &Fent, 1995; Hodson et al., 1996; Goksoyr et al., 1991). From large number of field studies, CYP1A enzymatic activities are often found to be enhanced in liver and gill of fish that have been exposed to oil contaminated water (Jönsson et al., 2006; Sarasquete & Segner, 2000). Both in vitro and in vivo studies found that the CYP1A induction level in mammals and fish are directly correlated with environmental contaminants, such as PAHs or PCBs (De Haan et al., 1996; Hornung et al., 1999). CYP1A induction has been used as a biomarker to monitor hydrophobic environmental pollutants for decades (Stegeman & Lech, 1991; Buhler & Wang-Buhler, 1998). Fish early life development studies showed that fish juveniles or embryos exposed to waterborne PAHs often developed symptoms such as pericardial and yolk sac edema, craniofacial and spinal deformities, hemorrhages, which is consistent with BSD syndromes (Carls et al., 2008; Hornung et al., 1999; Wolf, 1957). CYP1A induction is often detected in fish embryos.

3.1.1. Aryl hydrocarbon receptor and its ligands

The aryl hydrocarbon receptor (AhR) is a particular type of cytosolic transcription factor that mediates the regulation of a variety of genes; it is also a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) transcription factor family (Hankinson, 1995). It is activated through ligand binding and control the expression of several genes, including CYP1A. The detailed pathway was introduced in Chapter 1. Structure-activity studies indicate ligand often

required a planar structure to bind AhR, therefore some of halogenated aromatic hydrocarbons (HAHs) and PAHs are as strong AhR inducers (Hahn, 1998).

3.1.2. Polycyclic aromatic hydrocarbons - AhR inducers and inhibitors

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. PAHs could enter the aquatic environment through natural sources such as incomplete combustion of organic matters. But more often, as constituent of crude oil and refined mineral oil, PAHs enter the environment also through anthropogenic sources, such as crude oil and petroleum-related products discharge. Based on their toxicity, US EPA characterized 16 priority PAHs with ring numbers ranged from 2 to 6. From extensive case studies of these 16 PAHs, it is recognized that PAHs with less than 4 benzene rings are not CYP1A inducers; PAHs with four rings are weak or non inducers and with 5 and 6 rings are strong inducers (Fent & Batscher, 2000; Bols, 1999). Particularly, certain PAHs, such as fluroranthene, itself is not CYP1A inducers but rather CYP1A inhibitors (Willett et al., 1998). To be noted, the ability to induce CYP1A protein and catalytic activities for PAHs are species specific, the potency of PAHs may varies in different organisms.

PAHs with 5 and 6 rings are generally strong inducers of CYP1A. From extensive studies, dibenzo[a,h] anthracene (DBA), dibenzo[a,i] pyrene (DBP),BkF, BaP and Indenol [1,2,3-cd] pyrene (IdP) are typical strong inducers in both mammal and fish (Bols et al., 1999.; Fent & Batscher, 2000; Till et al., 1999). Even though the potency of these PAHs varies slightly in different species, their inducing concentrations are generally lower than other PAHs, while the induced enzymatic activities are higher. The characteristic EROD dose-response curve for these inducers is biphasic or bell-shape, that is, the CYP1A enzymatic activities induced in cells increased at low concentration and followed by deceased activities at high concentrations. In

contrast, the level of immunodetectable CYP1A protein, determined by either ELISA or Western blotting, correlated with the exposure concentration (Fent & Batscher, 2000; Levine & Oris, 1999; Till et al., 1999). Therefore, at high concentrations, 5 and 6 rings PAHs are more likely to reduce EROD activity by inhibiting CYP1A enzymatic activities, rather than interruption at the transcriptional or translational level.

PAHs with 3 or 4 benzene rings, including Fluorene (Fluor), anthracene, pyrenes, chrysenes, dibenzothiophenes (DBT), Fl and Benz[a]anthracene (BA), are non inducers or able to induce limited EROD activity, depending on the testing species (Bols et al., 1999; Bosveld et al., 2002; Machala et al., 2001). No inhibitory effects were observed in cells co-treated with BkF and fluorene or anthracene, which is neither CYP1A inducer nor inhibitor. Furthermore, many in vivo and vitro studies found that fish or rat hepatocytes co-treated with certain PAHs such as DBT, chrysenes, and Fl and a strong CYP1A inducer have decreased or no CYP1A enzymatic activities (Fent & Batscher, 2000; Wassenberg et al., 2005; Willett et al., 1998). It has been proven that these PAHs could reduce CYP1A activity either by inhibiting the enzymatic activity as a non-competitive inhibitor or down regulate the CYP1A expression or both. For instance, in Willett's et al. study, Fl is a non-inducer in rat hepatoma cells. When dosed with BkF and Fl, the CYP1A activities reduced while the CYP1A mRNA and protein level in the cells remained the same with cells treated by BkF alone (1998). However, when *Fundulus* microsomes were treated with BaP and Fl, both CYP1A protein level and enzymatic activity were decreased comparing with microsomes exposed to BaP alone (Willett et al., 2001). The contradictory results in two testing species indicated the underlying mechanisms for certain PAHs to exert toxicity could be multiple and vary between cell types and species.

Interestingly, the dose-response curves of oil mixture tested in fish or rat cell lines are biphasic, which proved that the toxicity of crude oil or petroleum products is mainly determined by their PAH composition, particularly the 5 and 6-ring PAHs (Huuskonen et al., 2000; Vrabie et al., 2009). However, when monitoring oil-like environmental pollutant, it is important to detecting both CYP1A enzymatic activities as well as the protein expression.

3.1.3. CYP1A induction measurement -EROD activity and Western Blot

All steps of the CYP1A induction cascade could be used to monitoring xenobiotics exposure to fish (Goksoyr et al., 1991). In Hahn's review, various methods using detecting CYP1A induction was summarized, i.e. the CYP1A gene activation could be detected by Northern blot or PCR; CYP1A expression could be immunologically detected by Western blot or ELISA; and the final products induced by xenobiotics could be measured by their catalytic activities (1998). Among them, measuring the enzymatic activity of CYP1A is the most fast, convenient way to detecting CYP1A induction.

Most studies of CYP1A induction in cell culture measure CYP1A enzymatic activity though the 7-ethoxyresorufin-o-deethylase (EROD) assay. It is a kinetic method which measures ethoxyresorufin-o-dealkylase enzymatic activity by converting the non-fluorescent substrate – 7-ethoxy resorufin into the fluorescent product, resorufin (Burke & Mayer, 1974). The CPYP1A catalytic activity could be analyzed conventionally by using microsomes prepared from fish tissues or directly measured in intact cells in cell cultures (Kennedy et al., 1995; Clemons, et al., 1996). However, due to the interactions between chemical contaminants and the enzyme and between PAHs, CYP1A catalytic activity does not always reflect the amount of CYP1A protein (Hahn et al., 1993). The catalytic activity of CYP1A largely depends on the chemical composition and the concentration of CYP1A inducers. Therefore, Immunochemical methods

such as Western blot and ELISA have been used in environmental monitoring on CYP1A-inducing pollutants. The biggest advantage of immunochemical assays is that they are sensitive to CYP1A inducers but are not influenced by inhibitors of CYP1A catalytic activity. Under most circumstances, the CYP1A expression could directly reflect the concentration of AhR agonists in the samples. This makes western blot or other immunochemical assays more suitable for evaluating contaminated samples such as oil spill, because contaminated samples are often a mixture with complex chemical composition.

3.1.4. Objectives

The objectives of this chapter is to assess the ability of HFO 7102 and its fractions, F2, F3, F3-1, F3-2 and F4 as HE-CEWAFs to induce EROD activity and CYP1A protein in RTL-W1.

3.2. Methods & Materials

All the oil samples used to evaluate CYP1A induction in RTL-W1were the same as used in Chapter 2. All the chemicals listed in this section were purchased from the same companies as used in Chapter 2. New chemicals required specially in this Chapter will be stated in below.

3.2.1. Cell culture maintenance

The cell culture was maintained as it described in in the Methods and Materials section of Chapter 2.

3.2.2. Preparation of Corexit 9500 and HE-CEWAFs of HFO 7102 and its fractions

Corexit were serially diluted in L-15 with 5% FBS. The highest Corexit concentration in culture was 0.05%.

HFO 7102 and its fractions were prepared as HE-CEWAF stock solutions as described in the Methods and Materials section of Chapter 2. The HE-CEWAFs were serially diluted in L-15 supplemented with 5% FBS.

3.2.3. Exposure of RTL-W1 to Corexit 9500 and HE-CEWAFs of HFO 7102 and its fractions

Confluent monolayer of RTL-W1 plated in 96-well tissue culture plates was used to study the CYP1A induction. The subcultivation procedures were the same as described in section 2.2.2, except for the FBS concentration in cell culture medium. Instead of using L-15 with 10% FBS, cells were plated in 96-well plate with L-15 with 5% FBS and 1% penicillin and streptomycin for three days.

At confluency, cells were exposed to Corexit 9500 or HE-CEWAFs. The exposure procedures were described in section 2.2.4. Cell with no treatments was as the negative control; a separated plate exposed to tetrachlorodibenzo-*p*-dioxin (TCDD; Wellington Laboratories,

Guelph, ON, Canada) was as the positive control. The exposure was stopped by removing

Corexit or HE-CEWAF dilutions and each well was rinsed by 50 µl PBS (pH=7.6).

3.2.4. EROD activity measurement

The schematic flowchart was shown in Appendix C Figure C.2. After exposure, the

microplates were assayed for 7-ehoxyresorufin-*o*-deethylase (EROD) activity directly. 7-

ethoxyresorufin (7ER), a non-fluorescent substrate, was added into each well. In the cells, the

substrate, 7ER, was converted into the resorufin, a fluorescent product by CYP1A protein. The

entire assay was run for 45 minutes and the product was measured with a fluorometric plate

reader, the CytoFluor 4000. Resorufin was excited at 530 nm and emission measured at 595 nm.

EROD activity was expressed as (pmoles resorufin / mg protein / min). The fluorescent

reading of resorufin was converted to pmoles using resorufin standard curves, which were

generated periodically. The protein amount in each well was determined with fluorescamine.

These steps were done as outlined in step by step fashion by Ganassin et al. (2000).

3.2.5. Western blotting for CYP1A protein

In order to collect enough protein for analysis, RTL-W1 were plated in 24-well plate with

200,000 cells per well. At confluency, cells were exposed to 1 ml of either Corexit dilutions or

oil/fractions HE-CEWAFs for 24 hours. To terminate the exposure, Corexit or HE-CEWAFs

were removed and well rinsed by 500 µl PBS.

CYP1A expression in RTL-W1 induced by Corexit or oil/fractions HE-CEWAFs were

analyzed by Western blotting. It was done as described in Clemons et al.(1972) and modified as

Hahn et al. described in their work (Hahn et al., 1972). The whole cell lysates treated with each

testing samples were employed for immunoblotting. To prepare lysates from cells grown in 24-

well plates, 20 µl of cold RIPA buffer with 0.1 µl of protease inhibitor was added to each well

and the plate was incubated at 4 ºC for 30 minutes with agitation. The cell lysates were transferred to 1.5 ml eppendorf tubes (one well per tube) and centrifuged for 10 minutes at 16,160 xg in the microcentrifuge. The supernatants were then transferred to newly labeled 1.5 ml eppendorf tubes. The concentration of total protein in each cell lysate was determined by Bradford assay.

Samples were analyzed by running denaturing gel electrophoresis on 4-12% polyacrylamide gradient gels. Proteins were then transferred from the gel onto nitrocellulose membranes. After blocked with 5% milk for one hour, the nitrocellulose membranes were incubated with the primary antibody (CYP1A, monoclonal mouse antifish, 1:3000; Cedarlane, Burlington, ON, Canada) for two hours, then with goat anti-mouse IgG linked with alkaline phosphatase (AP) (1:20000; Sigma-Aldrich, Saint Louis, MO, USA) for one hour. Color of the bands were developed by incubating membranes with the substrate solution, which was made by mixing 33 μl of 5-bromo-4-chloro-3-indolylphosphate (BCIP, Fisher Scientific) and 66 μl of nitro-blue tetrazolium chloride (NBT, Fisher Scientific) with 10 ml of pH 9.5 AP buffer.

3.2.6. Analyzing data

The raw fluorescent data was analyzed in Microsoft Excel. EROD activity was calculated as pmoles resorufin / mg protein / minute. Each data point was transformed into Log scale and were analyzed by non-linear regression with either biphasic, sigmoid or agonist stimulation model using the curve-fitting route. The EC_{50} values were calculated using a four-parameter logistic equation. To test the significant differences between results, One-way ANOVA with Tukey's test ($\alpha = 0.05$) was conducted. All graphs, statistical analyses and EC_{50} values were calculated using GraphPad Prism version 4.00 (GraphPad Sofrware, San Diego, USA).

3.3. Results

In this chapter, the ability of HFO 7102 and its fractions HE-CEWAFs inducing CYP1A activity in RTL-W1 was assessed by EROD assay. To investigate the inhibitory effects observed at high concentration of HE-CEWAFs, immunodetactable CYP1A protein amount induced by oil samples were measured by Western blot.

3.3.1. Ability of inducing EROD activity and CYP1A expression by Corexit 9500 in L-15/FBS

In order to rule out the possibility that the CYP1A induction is partially due to the Corexit in HE-CEWAF samples, RTL-W1 was first exposed to Corexit dilution alone for 24 h. CYP1A induction in RTL-W1 was screened by EROD assay and Western Blot. The results are shown in Figure 3.1. Panel A was the EROD activity in RTL-W1 induced by Corexit 9500. No EROD activities was observed in all the Corexit-treated cells. Panel B shows the EROD activity induced by TCDD, which serves as positive control. EROD activity induced by TCDD in RTL-W1 showed a dose-related increase until reached the maximum activity. In addition, maximum activity observed at concentrations ranging from 24.4-97.6 pM, resulting a top plateau formed in its dose-response curve. Panel C was CYP1A expression level in Corexit-treated cells. No detectable levels of CYP1A expression were induced in response to all the tested concentrations, which were higher than the actual Corexit concentration in HE-CEWAFs. Both Ponceau S stain of the membrane (bottom) and western blot (top) was shown in Panel C. The Ponceau S stain indicated that the protein loading of lanes was approximately equal. Exposure of RTL-W1 to the TCDD resulted in a strong induction of CYP1A expression, whereas no detectable levels of CYP1A expressed by RTL-W1 cells alone.
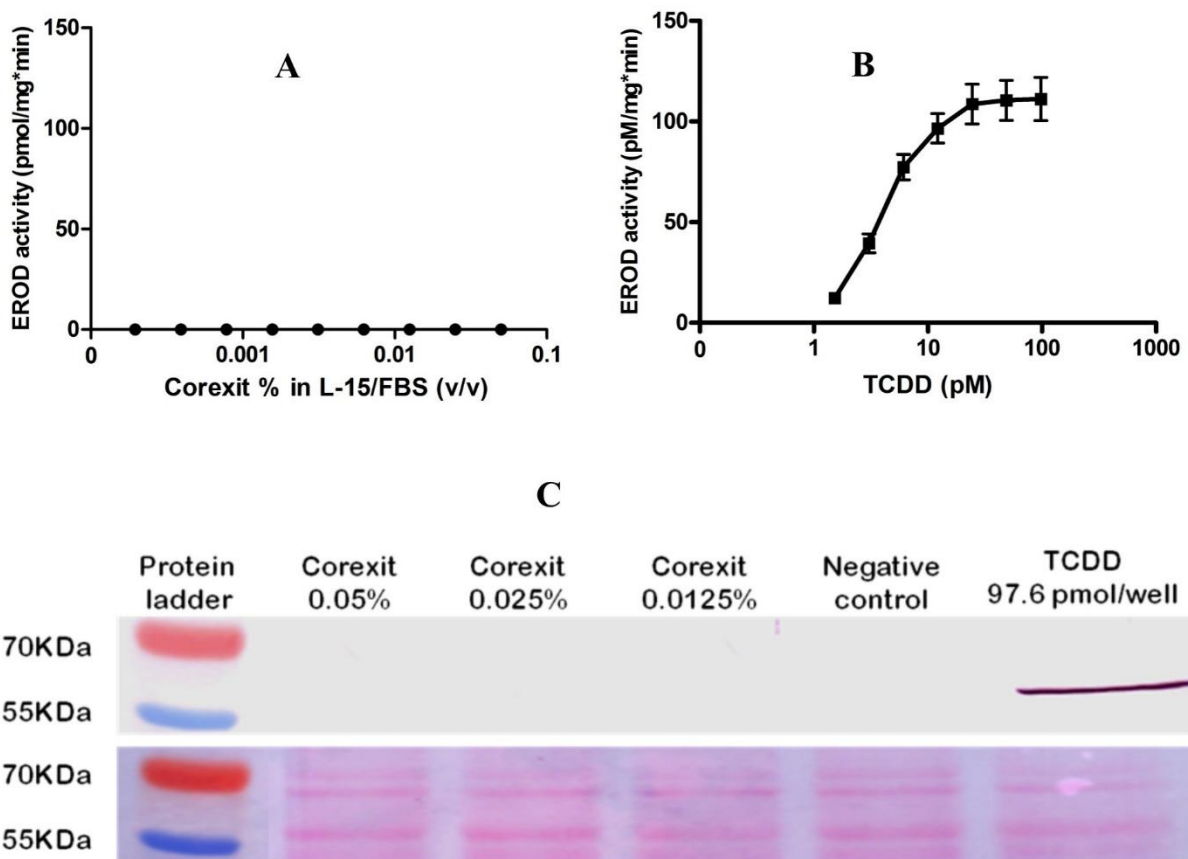
Figure 3.1. EROD activity and CYP1A protein induced by Corexit 9500 and TCDD. Cells were plated either in 96-well plate with an initial density of 50,000 cells/well (EROD assay) or in 24-well plate with an initial density of 200,000 cells/well (Western Blot). At confluency, RTL-W1 was treated with Corexit dilutions for 24 hours. Induced EROD activity and CYP1A protein were assayed immediately. The steps were outlined under Materials and Methods (A) EROD activity induced by Corexit 9500. (B) EROD activity induced by TCDD. Each point in A and B represents the mean activities (pmoles /mg*min) ± SD of 6 wells per dose. Each graph represent for three independent experiments (C) Immuno-detectable CYP1A protein. Both western blot and Ponceau S stain of the nitrocellulose membrane was shown. The first lane is protein marker and the last two lanes are negative control and cells dosed by TCDD, respectively. The three lanes in the middles shows the CYP1A induction in RTL-W1 treated with various Corexit dilutions. The amount of total protein in each lane was 20ng.

3.3.2. Ability of inducing EROD activity in RTL-W1 by HFO 7102 and its fractions

Whether or not that HFO 7102 and its fractions HE-CEWAFs have the ability to inducing CYP1A protein in RTL-W1 was determined by EROD assay. Monolayers were exposed to HE-CEWAFs dilutions of HFO 7102 and its fractions: F2, F3,F4, F3-1 and F3-2, for 24 hours and the catalytic activities of CYP1A protein was measured and expressed as EROD activity (pmoles /mg*min). Dose-response curve was constructed based on data collected by CytoFlour, as it shown in Figure 3.2.

HFO 7102 and all its fractions collected in Phase II and Phase III have the ability to induce EROD activity in RTL-W1. However, as AhR agonists or CYP1A inducers, their capacities and efficiencies of inducing EROD activities in RTL-W1 vary, which result in different types of dose-response relationships. EROD induction of HFO, F2, F3 and F3-1 were biphasic, which was increased and reached the maximum activity at lower concentrations and declined thereafter; EROD induction of F4, the asphaletane fraction, was sigmoidal, which increased to reach the maximum activity and maintained there at concentrations around 1 mg/ml. F3-2, however, as a wax residue, has limited ability inducing EROD activity and only when exposed to high concentrations (i.e. 20 mg/ml). Several possible scenarios could be the causes of biphasic curve, which will be further discussed in section 3.4. To compare their potencies and efficiencies as CYP1A inducers, EC_{50} values and the maximum EROD activity for each oil or fraction sample were estimated and listed inTable 3.1.
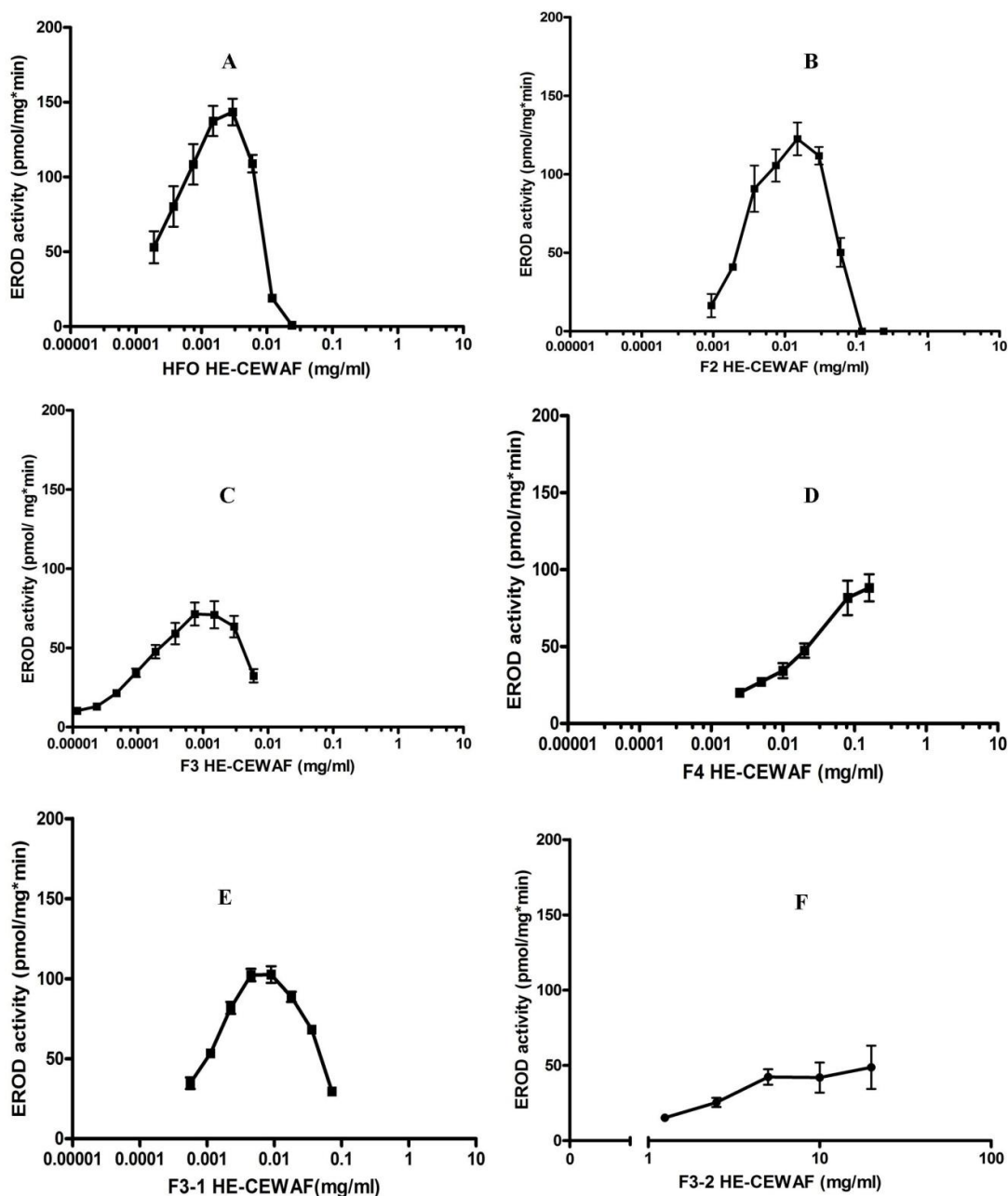
Figure 3.2. EROD activity induced in RTL-W1 by HFO 7102 or its fractions HE-CEWAFs.
96-well plates were plated with RTL-W1 cells with an initial density of 50,000 cells per well and cultured in L-15 supplemented with 5% FBS for 3 days. Freshly made oil/fractions HE-CEWAFs were used to expose RTL-W1 for 24 hours. The induced EROD activity was measured immediately after exposure, as it was outlined in Methods & Materials. Each point in every panel represents the mean activities (pmoles/mg*min) ± SD of 6 wells per dose. Each panel represents a typical dose-response curve for RTL-W1 treated by the following oil HE-CEWAFs: (A) HFO 7102 (B) F2 (C) F3 (D) F4 (E) F3-1 (F) F3-2

Table 3.1. The potencies and efficiencies of EROD induction in RTL-W1 by oil/fractions HE-CEWAF

Sample[1]	EC_{50} (mg/ml)[2] ± SD (n)	Maximum EROD activity (pmoles /mg*min)[3] ± SD (n)	Curve fit
HFO 7102	0.00498 ± 0.00432 (4)	123.2 ±30.9 (4)	Biphasic
F2	0.00503 ± 0.00295 (4)	92.3 ± 22.58 (4)	Biphasic
F3	0.00009 ± 0.00006 (4)	102.5 ± 39.77 (4)	Biphasic
F4	0.02467 ± 0.00913 (3)	84.2 ± 12.16 (3)	Sigmoidal
F3-1	0.00047 ± 0.00015 (4)	72.3 ± 25.10 (4)	Biphasic
F3-2	2.862 ± 0.061 (2)	43.9 ±3.09 (2)	Agonist stimulation

[1]*HFO 7102 is the parent oil; F2, F3 and F4 were collect in Phase I of EDFA method; To the most PAH-rich fraction, F3, two sub fractions, F3-1 (PAH rich) and F3-2 (wax residue) were separated by EDFA Phase II extraction*

[2] *EC_{50} value is the concentration eliciting 50% of the maximal response; the mean of EC_{50} estimated from either three or four trials along with their SD were listed in the table*

[3] *The maximum level of EROD activity represents the efficiency of induced CYP1A protein; the average EROD activities was also estimated from three or four independent experiments, along with the SD*

[2,3] *Every single EC_{50} value and the maximum EROD activities used to estimate the average values were calculated by GraphPad Prism 4.00*

EC_{50} values and the maximum EROD activity for each oil/fraction HE-CEWAF were estimated in order to determine their potencies and efficiencies. Comparing the numbers listed in Table 3.1, the potencies of HFO and its fractions HE-CEWAFs are: F3 (0.00009 ± 0.00006 mg/ml; n=4) ≈ F3-1 (0.00047 ± 0.00015 mg/ml; n=4) > F2(0.00503 ± 0.00295 mg/ml; n=4) ≈ HFO 7102 (0.00498 ± 0.00432 mg/ml; n=4) > F4 (0.02467 ± 0.00913 mg/ml; n=3) > F3-2 (2.862 ± 0.061; n=2). By comparison, the potencies of F3 and F3-1 are similar; F3 is approximately 100-fold potent than F2 and HFO; and 1000-fold potent than F4. Yet, under different concentrations, the maximum level of EROD activities induced by HFO and F2, F3, F4 and F3-1 are similar. F3-2 has limited activity. Analyzing both EC_{50} values and maximum EROD induction by One-way ANOVA, F3-2 is the least potent, F4 is significantly less potent than the rest of oil and fractions; there is no significant difference of Maximum EROD inductions between HFO and its fractions ($p < 0.05$). Nonetheless, the fact that the statistical analysis is contradictory with the experiments raw data could be due to weak statistic power caused by small sample size (n=4).

3.3.3. CYP1A induction in RTL-W1 by HFO 7102 and its fractions

In order to explore the possible causes of inhibition of EROD activity under high concentrations of several oil/fractions HE-CEWAFs (HFO, F2, F3 and F3-1), duplicated plates were used to detecting the CYP1A expression in RTL-W1. Selected concentrations of each sample's HE-CEWAFs, which cover 1000-fold range, were used to expose the cells. After 24 hours, cell lysates were collected and CYP1A protein was detected by western blot. Cells with no treatment is as the negative control; cells dosed with 97.6 pM TCDD is as the positive control. Both membrane (top) and Ponceau S stain (bottom) for each sample were shown in Figure 3.3.

In Figure 3.3, HFO 7102 and all its fractions have the ability to induce CYP1A expression in RTL-W1. Except for the protein ladder at the first lane, each band on the membrane represents detectable CYP1A and the darkness of the band is positively correlated to CYP1A expression level. That is, the darker the band is, the higher the CYP1A expression level is. Within each treatment, the CYP1A expression level increased with increasing HE-CEWAF concentrations, or more specifically, the CYP1A inducers in HE-CEWAFs. There is no doubt that F3 induced the most CYP1A and F3-2 induced the least. For the rest of oil and fractions, further calculations are needed in order to determine their potencies. Specially, for HFO, F2, F3 and F3-1, the CYP1A protein expression induced at high concentrations, which there is no EROD activity or declined EROD activity, is higher than the protein induced at concentrations, which have maximum EROD activity (HFO 7102 0.01mg/ml vs 0.001mg/ml; F2 0.1mg/ml vs 0.001mg/ml; F3 0.01mg/ml vs 0.001 mg/ml; F3-1 0.1 mg/ml vs 0.01 mg/ml).

The concentrations of inducing detectable level of CYP1A expression vary between HFO and its fractions: HFO 7102 (0.001 mg/ml), F3 (0.001 mg/ml), F3-1 (0.001 mg/ml), F4 (0.001 mg/ml), F2 (0.01 mg/ml), and F3-2 (7.5 mg/ml). Since only selected concentrations were used to expose cells, the practical concentrations that are able to induce detectable level of protein may be lower than the experimental set-up concentrations. Moreover, all the oil/fractions HE-CEWAFs produced a dose-dependent increase in CYP1A expression. Especially, for HFO 7102 and F2, F3 and F3-1, the elevated protein level at high concentrations is contradictory with their biphasic dose-response curves. After all, comparing within each membrane, as a strong AhR inducer, TCDD induce the most.
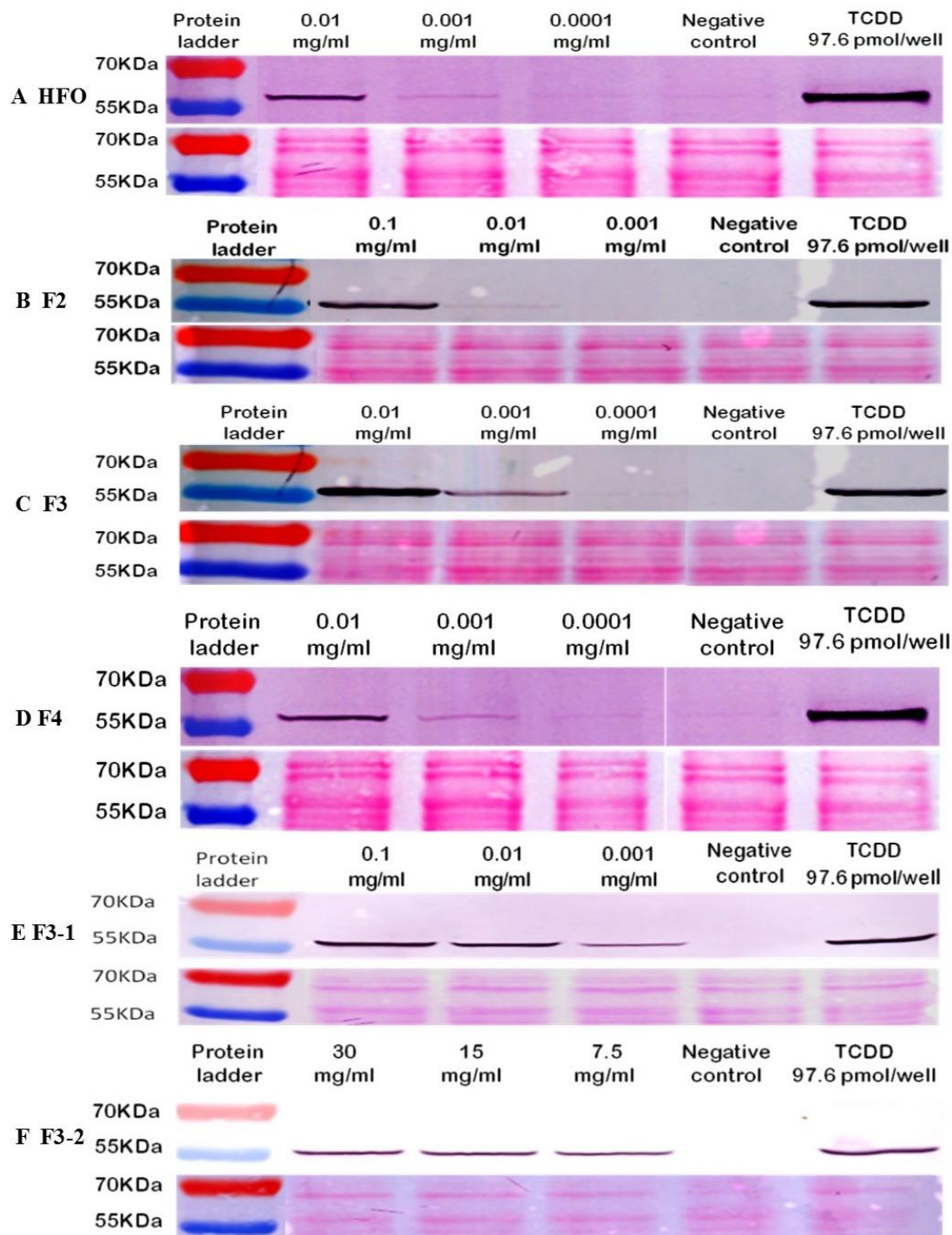
Figure 3.3. CYP1A expression in RTL-W1 induced by oil/fractions HE-CEWAFs. *Cells were treated as described in the legend to Figure 3.1. Cell lysates were separated by SDS-PAGE and the CYP1A expression were detected by monoclonal antibody, with detailed outlines described in*

section 3.2.4. The detected nitrocellulose membranes for each sample was scanned and both the membrane and its Ponceau S stain was shown. Panels from top to bottom illustrate the CYP1A protein amount induced under different concentrations of oil/fractions HE-CEWAFs: (A) HFO 7102 (B) F2 (C) F3 (D) F4 (E) F3-1 (F) F3-2. For each panel, first lane was loaded with protein standard; the last two lanes are negative control (cells with no treatment) and positive control (Cells exposed to 97.6 pM TCDD), respectively. In between, lane 2-4 represent lysates of cells dosed with the following concentrations of HE-CEWAFs: (A) HFO 7102: 0.01mg/ml, 0.001mg/ml, 0.0001 mg/ml; (B) F2: 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml;(C) F3: 0.01 mg/ml, 0.001 mg/ml, 0.0001mg/ml; (D) F4: 0.01 mg/ml, 0.001mg/ml, 0.0001 mg/ml; (E) F3-1: 0.1 mg/ml, 0.001 mg/ml, 0.0001 mg/ml; (F) F3-2: 7.5 mg/ml, 15 mg/ml, 30 mg/ml. All the inducing concentrations were marked on the top of each blot.

3.4. Discussion

The ability to induce CYP1A by HFO 7102 and its fractions in RTL-W1 was assessed enzymatically and immunochemically. Both EROD activity and Western blot suggested HFO and its fractions are able to induce CYP1A expression but their potency and efficiency are varied. In addition, to exclude the interference introduced by the use of Corexit, CYP1A induction in Corexit-treated RTL-W1 was also measured by EROD assay and Western Blot.

3.4.1. The CYP1A induction by Corexit 9500 in RTL-W1

The ability of inducing CYP1A by Corexit 9500 was first verified in Section 3.3.1. RTL-W1 in 72-well plate were exposed to different Corexit dilutions, with tested concentration ranging from 0.0001% - 0.05%. After 24 hours exposure, CYP1A enzymatic activity was examined by EROD assay. As the dose-response curve showed in panel A of Figure 3.1, Corexit 9500 does not have the ability to induce CYP1A activity within concentrations ranging from 0.0001%-0.005%.

The capacity of Corexit 9500 inducing CYP1A protein was evaluated by western blot. Cells were exposed to three Corexit dilutions: 0.05%, 0.025% and 0.0125%. After 24 hours, cell lysates were separated based on their molecular weight and transferred onto membrane and the protein expression was detected by monoclonal mouse antifish antibody. From the membrane showed in Figure 3.1 panel C, no CYP1A induction was observed in any of these three concentrations. On contrast, lysates of cells dosed by TCDD appears a strong CYP1A induction. In addition, lysates of cells with no treatment did not have CYP1A induction. Thus, Corexit 9500 does not have the ability to induce CYP1A protein in RTL-W1 neither.

In conclusion, within testing concentrations, no EROD activity was observed in cells exposed to Corexit 9500. In the meanwhile, no CYP1A induction were detected under high Corexit

concentrations. Therefore, Corexit 9500 seems do not contain any AhR agonists or CYP1A inducers. Furthermore, the practical Corexit concentrations in oil/fractions HE-CEWAFs are much lower than the testing concentrations here, the presence of Corexit should not have additive affects the CYP1A expression and catalytic activities induced by oil or its fractions HE-CEWAFs. However, the use of Corexit increased the bioavaibility and PAHs uptake were well documented in several studies (Ramachandran et al., 2006; Ramachandran, 2003; Schein et al., 2009).

3.4.2. The ability of HFO 7102 and its fractions HE-CEWAFs inducing CYP1A activities in RTL-W1

Testing with EROD assays, HFO 7102 and all its five fractions HE-CEWAFs have different ability to induce EROD activities in RTL-W1. The potency of these HE-CEWAFs could be ranked based on their mean EC_{50} values: F3 (0.00009 mg/ml $\pm$ 0.00006) $\approx$F3-1 (0.00047 mg/ml $\pm$ 0.00015) > HFO7102 (0.00498 mg/ml $\pm$ 0.00432) $\approx$ F2 (0.00503 mg/ml $\pm$ 0.00295) > F4 (0.02467 mg/ml $\pm$ 0.00913) >F3-2 (2.862 mg/ml $\pm$ 0.061).

The ranking of potencies for all the oil HE-CEWAFs is approximately consistent with the total PAH (TPAH) concentrations in the whole oil or fractions (Appendix A Table A.2).This is because comparing to other compounds in the oil (saturates, asphaltenes and resins), PAHs are high-affinity ligands for Ah receptors (Hankinson, 1995). In vivo and in vitro studies reviewed by Jacob (2008) suggested that to a complex mixture such as hard coal combustion or used motor oil, PAHs are the main contributors to CYP1A induction. The conclusion also holds in our study. For example, comparing with other fractions, F3 and F3-1, which contained PAH the most (8.5% and 9.2 % respectively), can induce CYP1A activities at fairly low concentrations. Also, HFO (2.6%) and F2 (5.2%) contain similar, intermediate amount of TPAHs, which induced EROD

activities at higher concentrations. Except for PAHs, asphaltenes and resins were also reported as Ah receptor agonists (Vrabie et al., 2012). Therefore, F4, composed by asphaltenes, resins and small amount of high molecular weight PAHs (2.6%), also has the ability to induce EROD activity. But asphaltenes are much weaker inducers comparing to certain PAHs (BaP for example). At last, F3-2 (0.002%), the wax residue of F3, little TPAHs amount in the sample results in only limited EROD induction at very high concentrations.

Noted that the differences between EC_{50} values were analyzed by One-way ANOVA, F3-2 and F4 are significantly different with the others, however, the post test (Turkey test) reported no differences between EC_{50} values of the whole oil, F2, F3 and F3-1 HE-CEWAFs. The contradiction between observed results and statistical analysis could be due to large standard deviation between trials and the small sample size. As mixtures, the complex PAHs composition in HFO and its three PAH-rich fractions, F2, F3 and F3-1 would bring variables into the system, resulting in deviations between trials. On contrast, F4 and F3-2 contains much less CYP1A inducers (i.e. PAHs), which could induced more stably. Therefore, the statistical power is too low to distinguish the potency differences between HFO and its PAH-rich fractions. This could be largely reduced by increasing sample size.

Except for F3-2, the efficiencies of CYP1A activities are similar between HFO and its fractions. Especially, F3, which contains CYP1A inducers the most, doesn't inducing higher enzymatic activities than other fractions. This is predicable since not only that F3 contains high concentration of CYP1A inducers which would interfere enzymatic activities, it also contains more 4 rings non-competitive inhibitors. Thus, CYP1A protein is induced the most by F3, but the catalytic activities was inhibited. Another possible cause for deceased CYP1A catalytic activity

could be competing catalytic sites between the inducer PAHs and the substrate 7-ehtrylresorufin at the active site. Similar results were also reported in Fent & Batscher's work (2000).

For PAH and alkane mixtures, the different types of dose-response curves for HFO and its fractions indicate that the composition of EROD inducers and/or inhibitors vary in samples. For each individual oil sample, inducers and/or inhibitors may affect not only AhR pathway, but a unique combination of pathways and mechanisms and interact with CYP1A directly or indirectly (Hahn, 1998; Hankinson, 1995; Jos et al., 2007). For instance, similar with TCDD, the dose-response curve of F4 is sigmoidal. The curve implies up to 1 mg/ml, CYP1A inducers rather than inhibitors dominate the EROD assay. When reached the limit of CYP1A enzymatic activity, the maximum activity was maintained at several concentrations. On contrast, the dose-response curves of HFO and its three PAH-rich fractions (F2, F3, and F3-1) are biphasic: at low HE-CEWAFs concentration, EROD activity in RTL-W1 was stimulated; at high HE-CEWAFs concentration, EROD activity was declined. In this case, both CYP1A inducers and inhibitors are present in oil or fractions mixtures. However, whether the inhibitors deactivate the CYP1A expression or simply inhibit the catalytic activities of CYP1A or even inhibitors indirectly affect CYP1A formation need to be further investigated. For instances, studies showed that not only AhR inhibitor, but also inhibitors of enzymes required for AhR pathway activation, such as tyrosine kinase or protein kinase C, will also reduce CYP1A induction (Backlund et al., 1997;Jos et al., 2007).

3.4.3. The ability of HFO 7102 and its fractions HE-CEWAFs inducing CYP1A expression in RTL-W1

To investigate the ability of HFO and its fractions to induce CYP1A protein in RTL-W1, the SDS-PAGE and western blot were employed to detecting the protein expression.

HFO 7102 and all of its fractions (F2, F3, F3-1, F3-2, and F4) have the ability to induce

CYP1A protein in RTL-W1, and the expression levels are all positive related to the exposure

concentrations. Within individual dose group, the relative CYP1A expression could be

determined by protein level induced by TCDD. Since all the cells as positive control were

exposed to fixed amount of TCDD (97.6 pM /well), the band density of each positive control,

could serve as standard. Thus, the relative potency of HFO and its fractions observed in Figure

3.3 is: F3 ≥ F3-1≥ F2 ≥ HFO 7102 >F4 > F3-2. However, the real potency need to be further

calculated based on the band density of individual oil sample comparing with their TCDD

positive control. Based on observation, it is no doubt that F3 is the most potent among HFO 7102

and all the other fractions.

Particularly, for HFO and its three PAH -rich fractions, comparing the panel A, B, C, E

between Figure 3.2 and Figure 3.3, neither their CYP1A expression level is consistent with their

EROD activities. That is, under high concentrations (beyond the maximum EROD activity),

increased CYP1A expressions in RTL-W1 were all coupled with diminishing EROD activities.

This result is comparable with several PAHs that are strong inducers such as BaP, BkF and IdP

(Machala et al., 2001). Several possible causes were bought up in pervious discussion, however,

based on these two facts, it can be concluded that the whole oil and its PAH-rich fractions all

contain different levels of both AhR inducers and CYP1A enzyme inhibitors. This conclusion is

reported in many studies focusing on single PAHs, such as BkF, FL, DBT. In addition, they are

all take a great portion in HFO and F2, F3 and F3-1 samples (Appendix A Table A.3), it is

possible that some of the PAHs could be also AhR inhibitors or substrate competitors or even

could activate other pathways, which inhibit CYP1A catalytic activities.

Interestingly, even though F3-1 has higher TPAH concentrations than F3, the protein amount induced by F3-1 HE-CEWAF was lower than F3 at the same concentration. The possible reason could be that not only CYP1A inducers but also CYP1A inhibitors, such as Fl, are concentrated in F3-1.This means that 1) as non-competitive inhibitors, the general CYP1A protein are induced less in F3-1; 2) as illustrated in Willett's et al. work, certain PAHs could down regulate the protein expression when present in a mixture (2001).

3.4.4. General conclusion

The general potency of HFO 7102 and its fractions were compared by their ability to induce CYP1A, which was measured as EROD activity and as the CYP1A protein, and based on the results, F3 would likely contribute the most to the toxicity of HFO 7102. However, interesting problems emerged. Under low HE-CEWAF concentrations of HFO 7102 and its fractions, EROD activity was induced before the CYP1A protein was detected by western blotting. However, at high HE-CEWAF concentrations, the immunochemical method avoided the inhibitory effects on the EROD assay of excess substrates and enzyme inhibitors in the test samples (HE-CEWAFs). This is illustrated with F2 as an example. At 0.01 mg/ml, F2 induced maximally EROD activity while the CYP1A expression was hardly detected. At 0.1 mg/ml, EROD activity was absent, likely due to a high amount of inhibitors, while high CYP1A levels were detected. The same results were observed with other PAH-rich samples, such as HFO and F3, F3-1 HE-CEWAFs. In general, the potency of HFO and its fractions determined by EROD assay and western blot are consistent and its order could reflect TPAH concentrations in every samples, as shown in Appendix A Table A.2 and A.4. The only exception would be F3-1, which is the PAH-concentrates of F3. With higher TPAH concentration, the potencies of F3-1 as determined by EROD assay and western blotting were both lower than F3. This could be because

that as well as CYP1A inducers F3-1 contains more inhibitors of CYP1A catalytic activity. Thus, when CYP1A is used as a biomarker for evaluating exposure of animals to environmental pollutants, EROD-inhibiting PAHs such as fluoranthene in the sample may reduce the EROD inducing potential of other PAHs and thereby influencing the PAH exposure assessments.

Chapter 4 Influence of Time of Exposure to Fraction 3 on CYP1A Induction in RTL-W1

4.1. Introduction

Persistent activation of the aryl hydrocarbon receptor (AhR) and the elevation of CYP1A levels might be necessary for the manifestation of diseases such as Blue Sac Disease (BSD). Thus whether the HFO 7102 increases CYP1A levels transitorily or permanently can help in assessing the long term risk of HFO 7102 to fish populations. If the main CYP1A inducers in HFO 7102 are PAHs, metabolism could influence the capacity of the HFO to induce CYP1A.

4.1.1. Polycyclic aromatic hydrocarbon (PAH) metabolism

Phase I and II metabolisms are important pathway in fish liver to steadily detoxify adverse substances. Fish have shown the capacity to metabolize and excrete PAH rapidly. A field study on Braer oil spill found that PAH level in fish tissue sampled from the pullulated area were not elevated 3 month after the incident, but the hepatic CYP1A activity was elevated (George et al., 1995). The rapid metabolism of PAHs is the main reason of the poor correlation between PAH concentration and CYP1A activity. In rainbow trout, the half-lives of some typical PAHs such as BaP, Fluorene, Pyrene and Chrysene are approximately 5 -7 days (Niimi & Palazzo, 1986).

The metabolism rate between CYP1A inducers are different. For instance, PAH are generally metabolized faster than dioxin-like compounds such as TCDD or PCB (Billiard et al., 2002). A parallel study conducted on Mirror Carp well described the influence of exposure time on CYP1A enzymatic activity. Cytochrome P450 1A activity was detected in the liver of Mirror Carp exposed to TCDD for 1 week and the activity lasted for 7 weeks without significantly decrease. However, both PAH level and CYP1A activity in the fish were detected to be the

maximum after exposing to PAHs for 2 days and the activity significantly deceased after 5 days. (Van der Weiben et al a & b., 1994). The different inducing time between TCDD and PAHs indicated that PAHs can be rapidly metabolized so that CYP1A activity declined within a shorter period than TCDD (Jones et al., 2000). Therefore, it is necessity of studying the time influence on CYP1A induction by oil-like contaminants, prior to evaluating results from biomornitoring programs in the aquatic environment (Celander et al., 1994).

4.1.2. Objectives

The main objectives of this chapter are to evaluate the effects of long term exposure to the F3 of HFO 7102 on the induction of EROD activity and expression of CYP1A protein in RTL-W1.

4.2. Methods & Materials

All the oil samples used to evaluate CYP1A induction in RTL-W1were the same as used in

Chapter 2. New methodology will be stated in this section.

4.2.1. Cell culture maintenance

The cell culture was maintained as it outlined section 2.2.1.

4.2.2. Exposure of RTL-W1 to Fraction 3 HE-CEWAFs for 5 different exposure periods

Five plates were used to assess the influence of time exposure to fraction 3 CYP1A

induction. F3 HE-CEWAF were prepared the same way as it was described in section 2.2.3. The

exposure procedures were performed as it outlined in section 2.2.4. However, instead of

exposure for 24 h, five exposure periods: 6 h, 24 h, 72 h, 120 h, 168 h, were tested.

4.2.3. Exposure of RTL-W1 to Fraction 3 HE-CEWAFs for 24 h followed by removal of inducers

Five plates were used to assess the influence of time exposure to fraction 3 CYP1A

induction. F3 HE-CEWAF were prepared the same way as it was described in section 2.2.3.

RTL-W1 were exposed to different concentrations of F3 HE-CEWAFs for 24 h in dark. The

exposure procedures were performed as it outlined in section 2.2.4. After the exposure, F3 HE-

CEWAF was removed and each well was well rinsed with 100 µl PBS. CYP1A protein level was

measured immediately after the removal of inducers or 24 h, 48 h, 72 h, 120 h, 144 h after

inducer removal.

4.2.4. Exposure of RTL-W1 to Fraction 3 HE-CEWAF and TCDD

F3 HE-CEWAF were prepared freshly in 15 ml effendorf tubes as it was described in section

2.2.3. In each dilution, fixed amount of TCDD was added to achieve a finial concentration of

97.6 pM/well. The mixtures were well mixed by vortexing. 200 ul of the mixture was applied to confluent monolayer in each well. RTL-W1 were exposed to the mixture for 24 h.

4.2.5. EROD activity measurement in RTL-W1

At the end of each exposure, the plate was measured as it stated in section 3.2.3.

4.2.6. CYP1A polypeptide measurement in RTL-W1

At the end of each exposure, cells were lysised and analyzed as it stated in section 3.2.4.

4.2.7. Analyzing data

Data was analyzed the same as described in 3.2.5.

4.3. Results

To study if the EROD activity and CYP1A expression level would change with prolongation of exposure time, the most potent fraction, F3, was studied in RTL-W1. Cells were exposed to F3 or TCDD for different time periods. After 6 , 24, 72,120 and 168 h exposure, the culture was either assayed for EROD activity or for CYP1A (P450 1A) polypeptide by Western blotting.

4.3.1. Influence of exposure time to Fraction 3 EROD activity in RTL-W1

Five multi-well plates were used to study the change in CYP1A catalytic activity (EROD) with prolonged F3 exposure periods, which were 6, 24, 72, 120, and 168 hours. EROD activity was measured at the end of each exposure period, and expressed as pmole resorufin /mg protein/ min (Figure 4.1). Both the EC_{50}s for EROD induction and the maximum EROD action after different exposure periods were calculated (Table 4.1).

EROD activity was induced in RTL-W1 after exposure to F3 HE-CEWAFs for 6 to 168 hours. The dose-response curves for all the five exposure periods were biphasic, as shown in Figure 4.1. Unlike TCDD, which had similar EC50 s for different exposure periods (12 to 120 hours; Bols et al., 1999), the dose response-curve for F3 was clearly shifted to higher concentrations after 7-day (168 hours) exposure. At high F3 concentrations ranging from 0.001 mg/ml-0.1 mg/ml, EROD activity showed only a slight change after 6 h. At the highest concentration (0.1 mg/ml), little EROD activity was observed, even after 168 h.

The potency of F3 HE-CEWAF as an inducer of EROD activity declined as the exposure time was extended. Table 4.1 summarizes the EC_{50} values of F3 HE-CEWAF for different exposure periods. The rank of potency is: 6 h (0.00008 ± 0.00005 mg/ml) > 24 h (0.00064 ± 0.00067 mg/ml) ≈ 72 h (0.00113 ± 0.00024 mg/ml) ≈ 120 h (0.00507 ± 0.00250 mg/ml) > 168 h (0.03568 ± 0.04901 mg/ml). By comparison, the EC_{50} for 6 h is approximately 10-fold lower

than 24 h and 72 h, 100-fold lower than 120 h and 1000-fold lower than 168 h. By One-way ANOVA analysis, EC_{50} value for 6 hours is significantly different with 168 h; EC_{50} values for 24 h, 72 h and 120 h are similar.
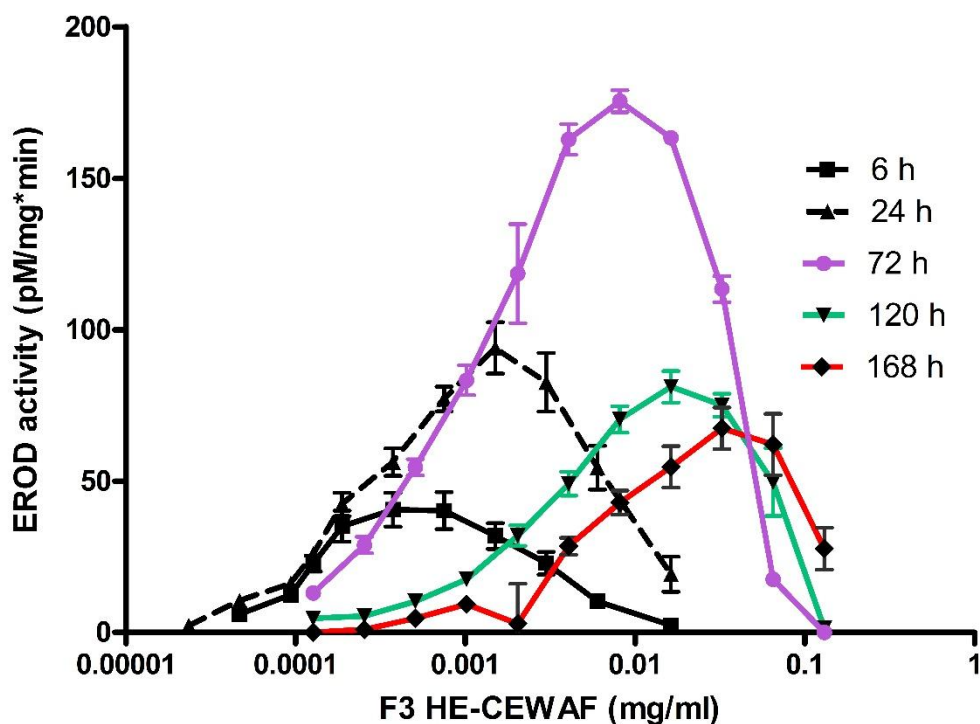
Figure 4.1.The time influence on CYP1A induction by F3 HE-CEWAFs. *RTL-W1 were plated in 96-well plate at an initial cell density of 50,000 cells per well. At confluency, cells were exposed to different F3 HE-CEWAF concentrations for 6 to 168 h. At the end of exposure, CYP1A induction were assessed by its enzymatic activity. Each concentration was repeated in 6 wells. The mean activity is presented as (pmole resorufin/ mg protein/min) along with their standard deviation. The graph represents one of three independent experiments.*

Table 4.1.The influence of exposure time on EROD activity induced by F3 HE-CEWAFs

Exposure time (h)	EC$_{50}$ (mg/ml) ± SD (n)	Max EROD activity (pmol/mg*min) ± SD (n)
6	0.00008±0.00005*(4)	50.75 ± 13.32 (3)
24	0.00064 ± 0.00067 (3)	97.41 ± 47.79 (3)
72	0.00113 ± 0.00024 (3)	120.72 ± 73.32 (3)
120	0.00507 ± 0.00250 (3)	91.25 ± 21.09 (3)
168	0.03568 ± 0.04901*(3)	68.01 ± 24.41 (3)

*EC$_{50}$ values measured at each exposure time were analyzed by One-way ANOVA; in each column, numbers with * are statistically different with each other*

4.3.2. Influence of exposure time to Fraction 3 CYP1A expression in RTL-W1

To test whether the levels of CYP1A protein would change the same way as CYP1A catalytic activity, RTL-W1 cultures were exposed to F3 HE-CEWAF for 6, 24, 72, 120, and 168 h. Cultures exposed to TCDD were the positive control; cultures with no treatment were negative control. The CYP1A protein was analyzed by western blotting; protein levels were calculated and expressed as percentage of protein amount induced by 97.6 pM TCDD alone

Figure 4.2 shows the immunodetectable CYP1A protein detected by Western Blot. First, the similarity between Ponceau S stain for all the analyzed nitrocellulose membrane suggested that protein amount loaded in each lane was approximately equal. This means that rather than unequal loading, the differences between band densities are caused by varied protein amount detected by CYP1A antibody. At all the testing concentrations, CYP1A expression in F3-treated cells seems decreased with the prolongation of exposure period. However, CYP 1A protein induced by TCDD increased over time. To further compare CYP1A expression for different exposure period, the immunoblotting was scanned and the bend density was estimated by Image J.

The raw data of each band density estimated by Image J was not shown here. This is because that CYP1A expression is primarily affected by AhR ligand such as PAHs or TCDD, other factors such as cell growth over time will also affect the induction. In addition, CYP1A expression induced by TCDD was increased with the prolongation of exposure period, it is reasonable to report the relative CYP1A protein amount instead of the apparent protein amount. Table 4.2 showed relative CYP1A products induced by F3 HE-CEWAF over time. The relative CYP1A product was expressed as the percentage of CYP1A band density induced by TCDD within the same exposure period. After the conversion, the graph representation of the relative

CYP1A protein induced by F3 HE-CEWAF with prolongation of exposure period is present in Figure 4.3.

All the tested concentrations of F3 HE-CEWAFs are able to induce CYP1A expression within a certain exposure period; the higher the dose is, the longer exposure time that cells are needed to reach maximum CYP1A expression level. (Figure 4.3). In Figure 4.3, the highest CYP1A induction for 0.0001, 0.001, and 0.1 were observed at the following exposure times: 6 h, 6 h, and 72 h, respectively. For 0.01 mg/ml, the maximum CYP1A induction was observed at 24 h exposure. In addition, at each individual dose, CYP1A protein amount reached the maximum and declined afterwards. For example, CYP1A expression induced at 0.001 mg/ml were increased at 6 and 24 h and decreased for longer exposure periods (Table 4.2). CYP1A amount induced at the other concentrations, 0.0001, 0.001 and 0.1 mg/ml were also decreased with the prolongation of exposure period. Comparing CYP1A expression in RTL-W1 with the same exposure period, the induced protein amount is positively correlated with the F3 HE-CEWAF, or TPAH concentration.
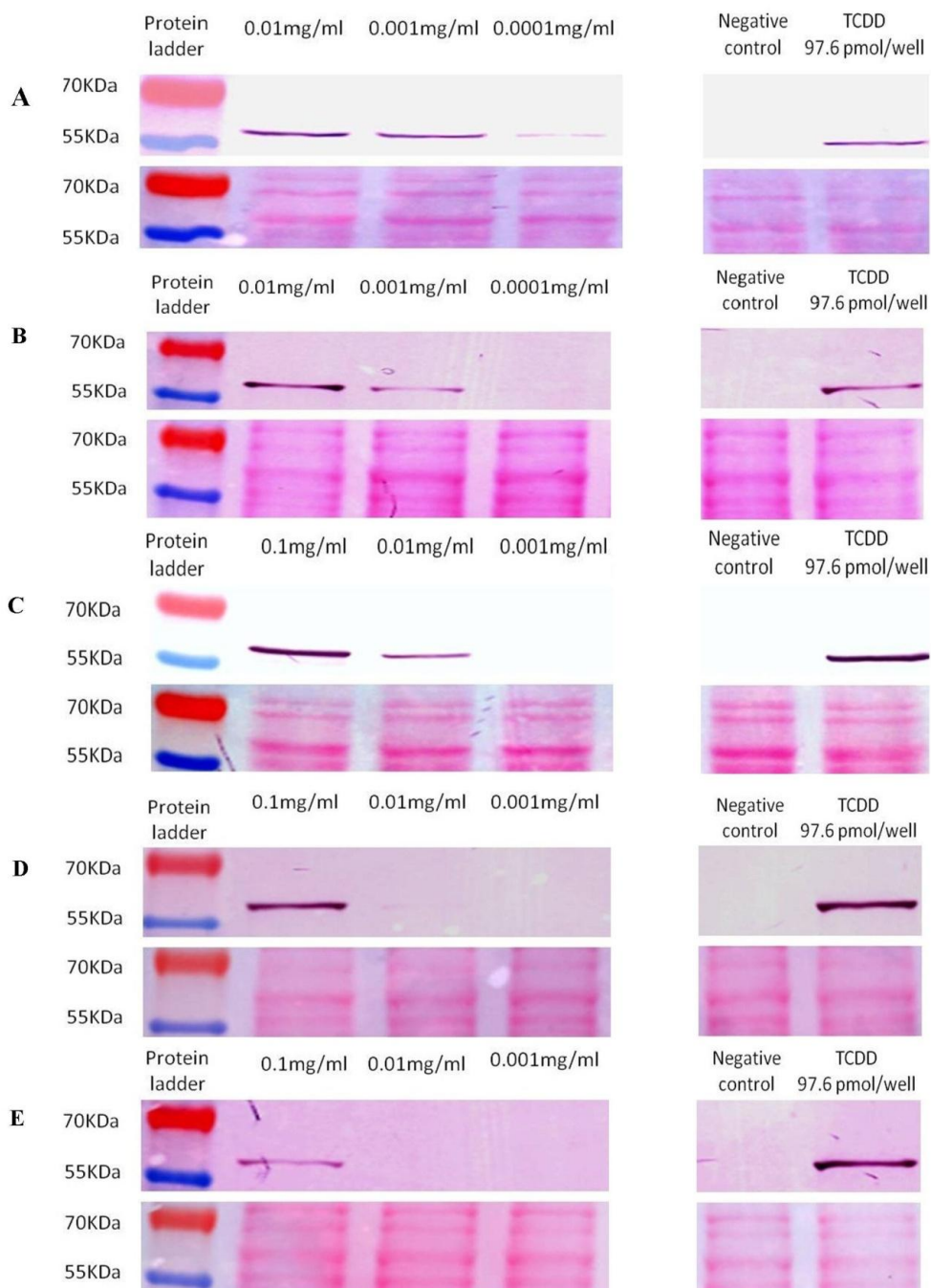
Figure 4.2 CYP1A expression induced by F3 HE-CEWAFs for 5 different exposure periods.
Cells were treated as described in the legend to Figure 4.1. At the end of each exposure period,
CYP1A protein was analysis by Western Blot as it outlined in section 4.2.6. Panels from top to
bottom represent CYP1A expression level for (A) 6 h (B) 24 h (C) 72 h (D) 120 h (E) 168 h. In
each panel, first lane was loaded with protein standard; the last second lane is negative control
(cells with no treatment) and the last is positive control (cells exposed to 97.6 pM TCDD). In
between, each band represents CYP1A amount with the induction concentration indicated above
respective membrane.

Table 4.2. The relative CYP1A protein amount induced by F3 HE-CEWAFs with various exposure periods

Exposure time (h)	CYP1A protein amount (% TCDD response alone) ± SD (n)			
	0.0001 mg/ml[*]	0.001 mg/ml[*]	0.01 mg/ml[*]	0.1 mg/ml[*]
6[♦]	12.53[a,b,i] ± 0.13 (3)	85.74[b,i] ±11.03 (3)	87.84[a,i,j] ± 9.55 (3)	n.d.
24[♦]	1.84[a,i] ± 0.05 (3)	36.60[a,i] ± 3.48 (3)	148.51[a,i,j] ± 10.20(3)	n.d.
72[♦]	n.d.	u.d. [a,i]	60.90[a,i,j] ±1.09 (3)	163.33[a,i] ± 2.39 (3)
120[♦]	n.d.	u.d. [b,i]	2.49[a,i] ± 0.39 (3)	135.90[a,b,i] ± 3.70 (3)
168[♦]	n.d.	u.d. [a,i]	u.d. [b,j]	49.37[a,b,i] ± 6.54 (3)

[♦] *Within each row, means compared by One-way ANOVA were significant (p<0.05)*
[*] *Within each column, means compared by One-way ANOVA were significant (P<0.05)*
[a-b] *means within each row the numbers with the same letter are significantly different*
[i-j] *means within each column, numbers with the same letter are significantly different*
n.d. means CYP1A protein was not tested with that specific combination of exposure period and concentration
u.d. means CYP1A protein was undetectable with that specific combination of exposure period and concentration

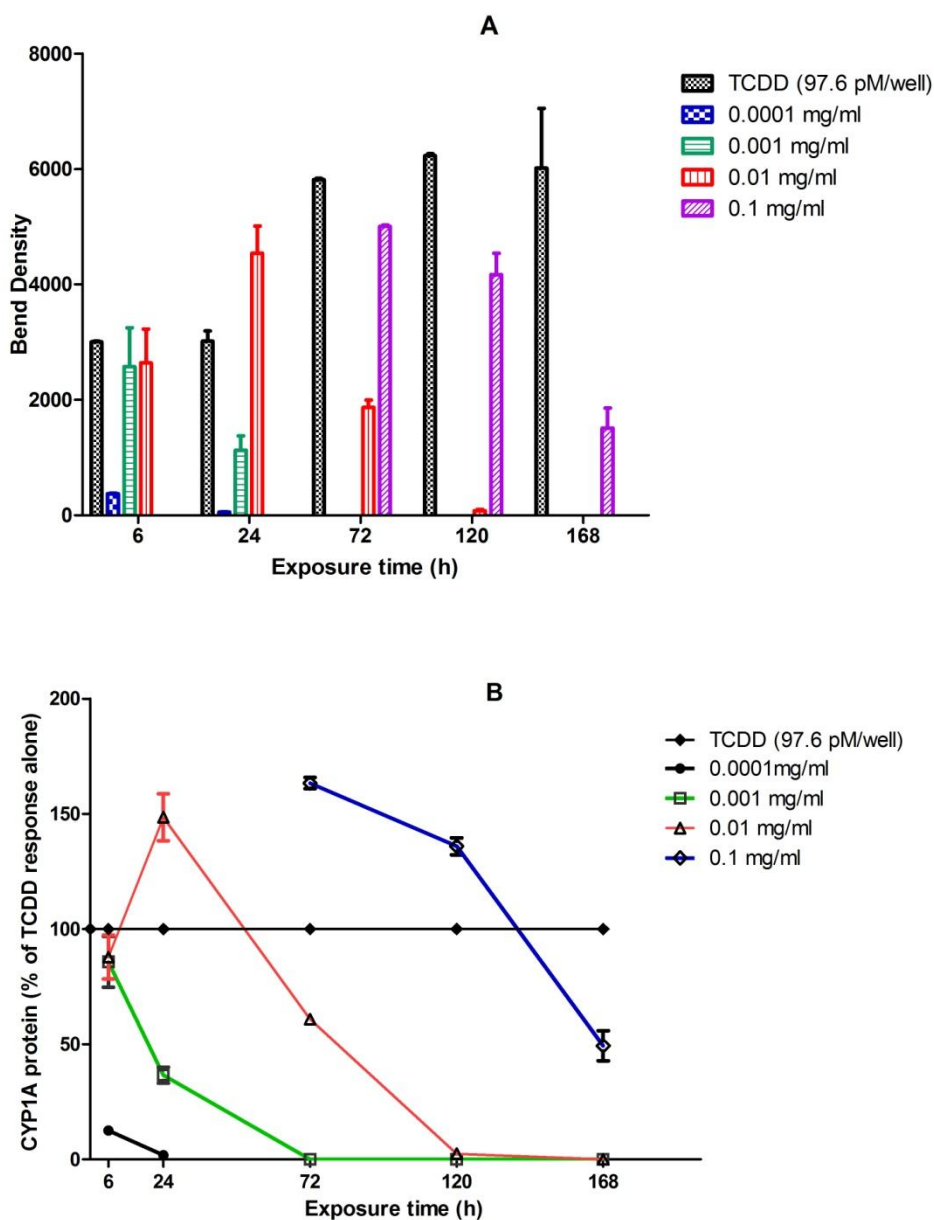**Figure 4.3. Graphic representation of band intensities of CYP1A induction by F3 HE-CEWAF with the prolongation of exposure period.** *Western blots for each exposure period in Figure 4.2 was scanned and the CYP1A protein amount was estimated based on their corresponding bend density. The protein amount was expressed as the percentage of protein induced by 97.6 pM TCDD alone. Each bar represents two independent experiments.*

4.3.3. CYP1A expression with time after the removal of F3 HE-CEWAF

To exclude the possibility that declined CYP1A protein expression with prolongation of exposure time is due to PAH partition out of culture media, immunodetectable CYP1A protein was measured over time after 24 h exposure to F3 HE-CEWAFs. Specifically, confluent monolayer was dosed by F3 HE-CEWAFs for 24 h then HE-CEWAFs was replaced by fresh media. The protein amount was measured by Western blot at 0, 24, 48, 72, 120, 144 h respectively after media change.

CYP1A expression was declined with prolongation of time. Similar Ponceau S stain for all the analyzed nitrocellulose membranes suggested that protein amount loaded in each lane was approximately equal. Over time, CYP1A protein induced by TCDD increased, even after the media removal. On contrast, CYP1A expression was declined at all HE-CEWAF concentrations after media change (Figure 4.4 A). To compare the CYP1A protein amount induced for different exposure period, the protein expression was converted into the percentage of CYP1A induced by TCDD with the same exposure period and showed in Figure 4.4 B. For the two intermediate concentrations (0.001 mg/ml and 0.01 mg/ml), after removed CYP1A inducers, CYP1A expression was decreased but continued to be expressed for 24 h and 48 h. At the highest concentration (0.1 mg/ml), however, after removed the inducers, CYP1A expression level was elevated in the next 24 h then decreased. Unlike the intermediate concentrations, CYP1A induced at 0.1 mg/ml was continued to be induced after remove the inducers for 144 h.
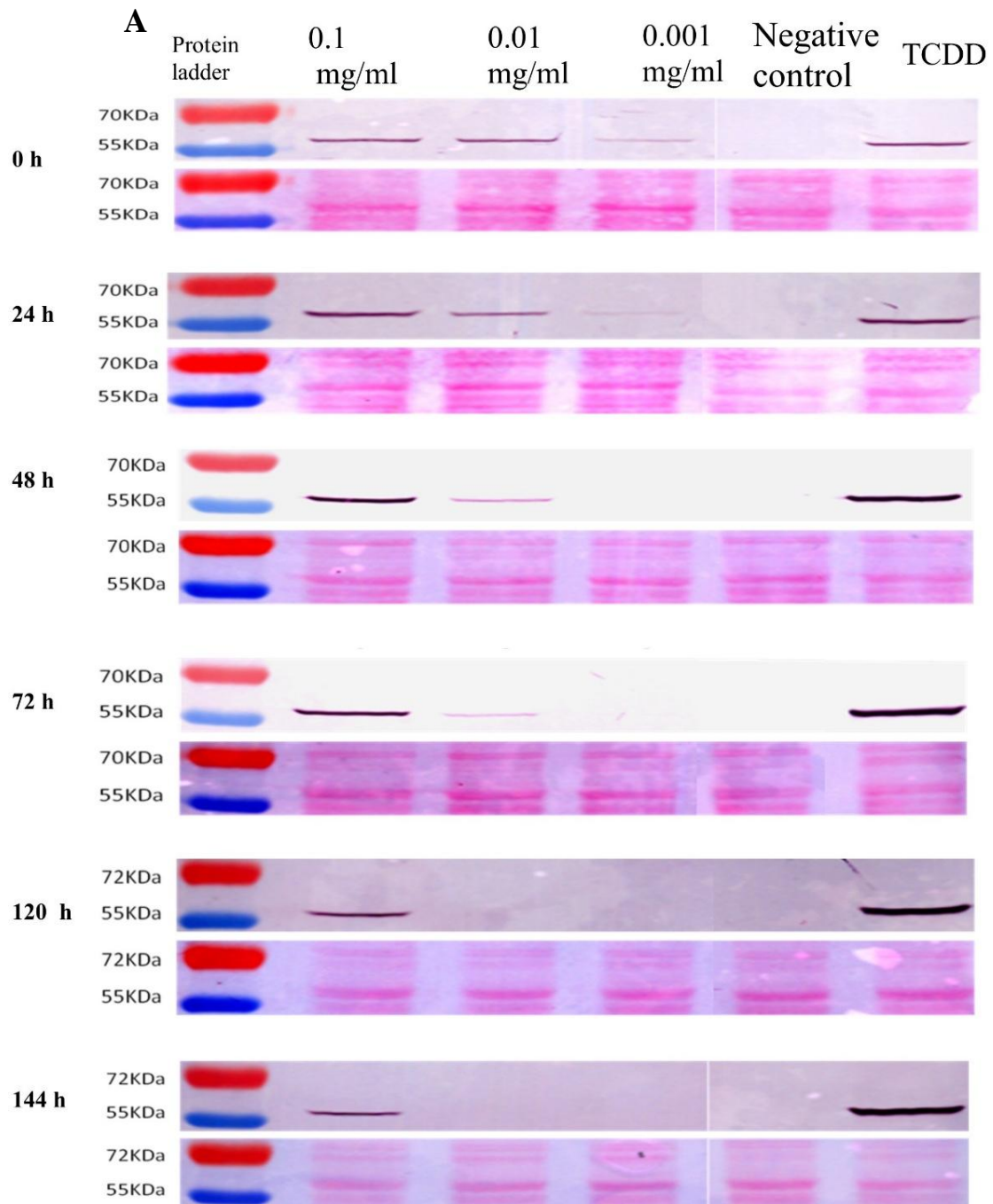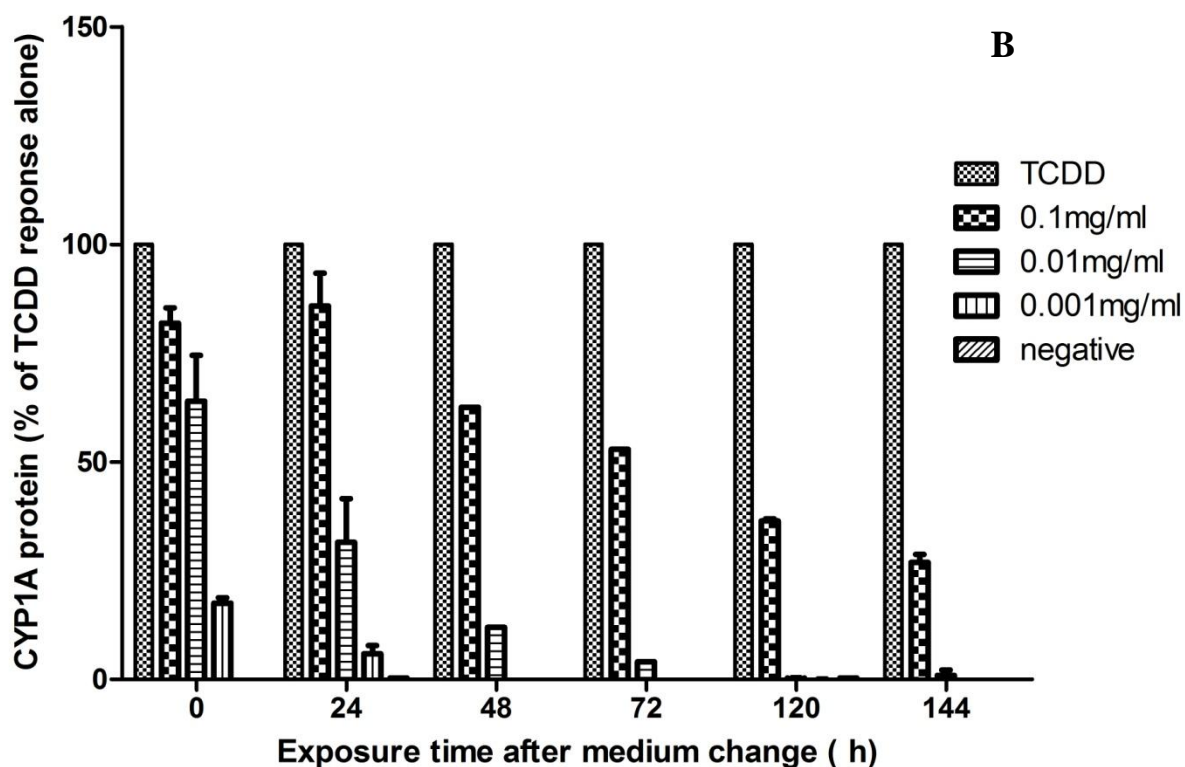
Figure 4.4. CYP1A expression induced by F3 HE-CEWAFs after the medium change. *RTL-W1 was plated for 3 days to reach confluency. Confluent monolayer in each well was dosed to F3 HE-CEWAFs for 24 h. At the end of exposure, F3 HE-CEWAFs were replaced by fresh media and protein in RTL-W1 was measure by Western Blotting immediately or cells were further incubated for 24, 48, 72, 120 and 144 h respectively followed by Western Blot. Panel (A) Immunoblot detection of CYP1A protein induced by F3 or TCDD after media change. Panel (B) Graphic representation of band intensities of immunodetectable CYP1A protein. Both graphs represent two independent experiments.*

4.3.4. CYP1A induction in RTL-W1 exposed to F3 HE-CEWAF and TCDD

RTL-W1 was exposed for 24 h to high concentration (0.03-1 mg/ml) of F3 HE-CEWAF together with a fixed amount of TCDD (97.6 pM/well). At the end of the exposures, both EROD activity and CYP1A protein levels were measured. The results for F3 HE-CEWAF and TCDD were compared to those induced by TCDD alone and expressed as the percentage of TCDD response alone.

The results are summarized in Figure 4.5. No or little EROD activity was observed in cells exposed to the mixtures of TCDD and F3 HE-CEWAFs, but high EROD activity was seen in cells exposed to TCDD alone (Figure 4.5 A). As measured by western blotting, CYP1A protein was induced by HE-CEWAF and TCDD together as well as by TCDD alone (Figure 4.5 B). CYP1A protein levels appeared similar in cultures with different combinations of HE-CEWAF and TCDD and with TCDD alone (Figure 4.5 B). To quantify this, each membrane from western blotting was scanned and analyzed by Image J. The relative CYP1A protein amount was calculated and plotted as shown in Figure 4.5 C. Analyzed by One-way ANOVA, except for the negative control, CYP1A levels in RTL-W1 exposed to TCDD alone and to a mixture of F3 HE-CEWAF and TCDD were not significantly different ($P>0.05$). Therefore, F3 HE-CEWAF did not interfere with the induction of CYP1A protein by TCDD, but did inhibit the catalytic activity (measured as EROD) of the induced CYP1A protein.
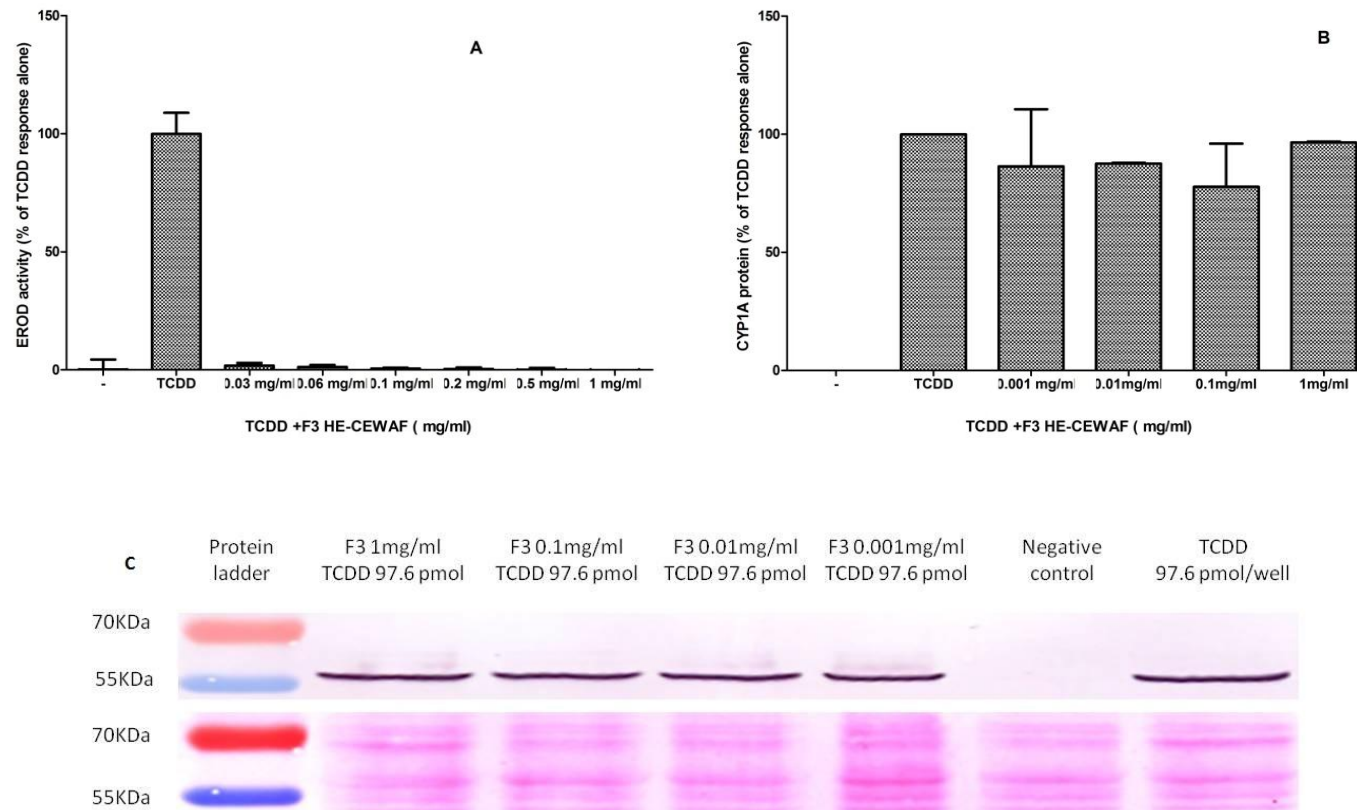
Figure 4.5. CYP1A induction in RTL-W1 exposed to F3 HE-CEWAF and TCDD. *RTL-W1 were plated in 96-well plate to for 3 days to reach confluency. Confluent monolayers was treated with fix amount of TCDD (96.7 pM) and various concentrations of F3 HE-CEWAFs for 24 hours. Cells with no treatment is as negative control; cells dosed with TCDD (96.7 pM/well) alone is as positive control. At the end of exposure, CYP1A induction was assessed by both EROD activity and Western blotting. (A) Inhibition of CYP1A catalytic activity in RTL-W1 dosed by F3 HE-CEWAF and TCDD (B) Graph representation of band intensity of CYP1A expression in RTL-W1 dosed by mixture of F3 HE-CEWAF and TCDD. CYP1A protein amount induced by F3 HE-CEWAF and TCDD is not significantly different with protein induced by TCDD alone, judged by One-way ANOVA (P=0.55) (C) CYP1Aexpression detected by Immunoblotting in RTL-W1 dosed by F3 HE-CEWAF and TCDD.*

4.4 Discussion

A time-course study was conducted with the PAH-rich fraction (F3) that was the most potent inducer of CYP1A in RTL-W1. The two sets of experiments showed that EROD activity and CYP1A protein expression changed with the prolongation of exposure time. EROD assay and Western blot were employed to track CYP1A induction. In order to further confirm the causes for CYP1A induction change over time, RTL-W1 were exposed to F3 and TCDD together or exposed to F3 for 24 h and then remove F3 HE-CEWAF for further exposure.

4.4.1 Influence of exposure time to Fraction 3 EROD activity in RTL-W1

The CYP1A activity induced by F3 HE-CEWAF is temporal. Even though the maximum activity were similar over time, for all the testing concentrations, their CYP1A activity was increased to the maximum level and declined thereafter. Also, RTL-W1 exposed to higher TPAH concentrations required a longer exposure period to reach the maximum activity. The pattern of CYP1A activity change over time is not exclusively but mainly due to the reduced amount of CYP1A-inducing compound and enzyme inhibitor. For instance, at low concentrations (0.0001-0.001 mg/ml), CYP1A activity was declined over time. This is because that CYP1A is sensitive enough to reach maximum induction quickly at these concentrations without inhibitory effects on its enzymatic activity. As PAHs in RTL-W1 was removed though Phase I and II metabolism, the CYP1A protein level decreased as well, which lead to the declined CYP1A activity. However, at high concentration (0.01-0.1 mg/ml), the reduced inducers amount recovered CYP1A catalytic activity. That is, for 6 hour exposure, little or no EROD activity was observed at concentration ranging from 0.01-0.1 mg/ml, with prolonged exposure periods, EROD activity was increased at these concentrations. As discussed previously, this could be due to the excess CYP1A inducers and inhibitors present in culture medium. With the prolongation of time, both inducers and

inhibitors was removed by PAH metabolism, which released the enzyme from inhibition state and caused elevated EROD activity at high concentrations with prolongation of exposure time.

The potency of F3 HE-CEWAFs was declined with prolongation of exposure time. EROD activity induced by F3 HE-CEWAF for 5 exposure periods was measured in 1000-fold range. The dose-response curves for all testing periods are biphasic. However, the potency of CYP1A activity, determined by EC_{50} values, was reduced over time. From Figure 4.1 A, EC_{50} for 6 h is 1000-fold less than it $EC_{50}s$ for 168 h. The analysis of variances tests also indicated that the EC_{50} values for 6 h and 168 h are significantly different, which proved that the dose-response curve shifted to the higher concentration of F3 HE-CEWAF over time. However, One-way ANOVA analysis indicated that the EC_{50} values between 6 h, 24 h, 72 h and 120 h exposure are similar. This could be due to large standard deviations between readings and small sample size (n=3). Several possible reasons such as the reduction amount of inducers, down regulation of CYP1A gene caused by AhR inhibitor, inhibition of CYP1A enzymatic activity and the competition between CYP1A inducers and EROD substrates could contribute together or separately to the shifting.

As a PAH mixture, there are more than one reasons lead to the CYP1A induction changes over time. The impaired CYP1A activity at high TPAH concentration for 6 or 24 h exposure could be due to Ah receptor inactivation and CYP1A down regulation by certain PAHs. In addition, except for PAH metabolism, partitioning could be another possible reason for the reduction of CYP1A inducers (PAH primarily) over time. The following experiments would be further confirm these possibilities.

4.4.2. Influence of exposure time to Fraction 3 CYP1A expression in RTL-W1

CYP1A protein was measured by western blotting for all the exposure periods. CYP1A protein levels showed a time and dose-related increase (Figure 4.3 B). By contrast, at high dose, the CYP1A catalytic activity as EROD activity declined. For example, EROD activities declined at concentrations from 0.001 – 0.01 mg/ml for both 6 and 24 h exposure, however, the CYP1A protein expression increased for the same concentrations for both exposure periods. Similar contradictory results for EROD activity versus CYP1A protein levels were also observed in cells exposed to 0.01-0.1 mg/ml for 72, 120 and 168 h exposure, respectively. The inconsistency between CYP1A induction and activity indicates that even though the CYP1A protein was expressed, the cytochrome P450 enzyme either loss its catalytic activity or the activity is inhibited by F3 HE-CEWAF. However, it is unlikely that the expressed CYP1A protein is not functional because the CYP1A activity could be recovered with prolongation of exposure (Figure 4.1).

CYP1A induction by F3 HE-CEWAF is also temporal. That is, with prolonged exposure period, CYP1A expression level was declined till zero. At 0.0001, 0.001 and 0.01mg/ml, the maximum CYP1A expression was detected at 6, 6 and 24 h exposure, respectively; with prolonged exposure period, the CYP1A expression level declined and no protein was detected at 24, 72, and 120 h exposure, respectively, as it was shown in Figure 4.3 B. Since F3 HE-CEWAF does not down regulate CYP1A gene expression, the protein level is directly correlated with inducer concentration. Thus, the declined CYP1A expression indicate that inducers concentration was reduced with prolonged exposure time and when inducer concentration is close to zero, the protein level is undetectable. As the CYP1A inducers is also CYP1A substrate, it is highly possible that the inducers are removed by Cytochrome P450 enzymes. However, it is not likely

or entirely due to PAH partitioning out of the culture medium. The reason will be stated in the next section (4.4.4).

In addition, unlike PAHs, CYP1A expression induced by TCDD was prolonged and dose-dependent increase. This is because that TCDD is a high-affinity ligands for AhR and has a slow metabolism rate in cells, which lead to a prolonged high-level of CYP1A induction. The differences of inducing time and pattern between TCDD and PAH mixture was also observed in human cell line (Jones et al., 2000).

4.4.3. CYP1A expression over time after medium change

To exclude the possibility that PAH amounts were reduced due to the PAHs partitioning out of media into insoluble particles, RTL-W1 was exposed to the same concentrations of F3 HE-CEWAF used in the previous experiment for 24 h, then the medium with HE-CEWAF was replaced with fresh media and measuring CYP1A expression at 0, 24, 48, 72, 120 and 144 h after the media change. At all the concentrations (0.001, 0.01 and 0.1 mg/ml), CYP1A protein amounts reduced but continued expressed with the prolongation of time. Particularly, at 0.1 mg/ml, CYP1A induction reached to the maximum at 24 h after removing inducer then declined. Since the PAH inducers had removed from the culture medium, the prolonged CYP1A expression was caused by PAHs (inducers) present in the cell. As it was discussed in the previous section, CYP1A protein expression is directly correlated with the inducer concentration. In this case, the declined CYP1A induction is caused by reduced concentration of PAHs in the cell. Thus, this result proved that 1) due to their hydrophibicity, PAHs could easily across cell membranes and bind to AhR 2) over time, the inducers (PAHs) will be removed by Phase I and Phase II metabolism. Ultimately, no CYP1A induction will be observed after all the inducers have been removed ,as it was illustrated in Figure 4.4. (No protein was detected in RTL-W1

exposed to 0.001mg/ml F3 HE-CEWAF at 24 h after medium change and 0.1 mg/ml at 120 h after medium change).

4.4.4. CYP1A induction in RTL-W1 by F3 HE-CEWAF and TCDD together

In as much as the F3 might contain compounds that inhibited as well as activated the AhR in RTL-W1, HE-CEWAF and TCDD were tested together. HE-CEWAF and TCDD together induced CYP1A protein to the same level as with TCDD alone. This suggests that there was no inhibitor in F3 that inhibited AhR activation by TCDD. As well, the result suggests that the CYP1A induction had been maximized by TCDD and AhR agonists in F3 were much less effective than TCDD so induction was not further increased. However, the induction of EROD activity was completely blocked. This could be due to compounds in F3 that interfere with the measurement of the catalytic activity of CYP1A as EROD activity. Such inhibitors could be carried over into the EROD assay after the 24 h exposure to F3 HE-CEWAF and TCDD. This is because the assay is done with live intact cells and compounds might accumulate in the cells during the exposure period and remain inside despite the cells being rinsed before the EROD assay. Potential inhibitors are small PAHs, such as fluoranthene (Fl). Fl has been shown to inhibit CYP1A catalytic activity in mammalian cells (Willett et al., 1998). Whether such compounds interfere with EROD induction by F3 HE-CEWAF alone is an interesting question. The interaction between PAHs within HE-CEWAF might be different than the interaction(s) with TCDD. In mammalian cells, Fl interacted differently with BaP than Fl did with TCDD in the induction of CYP1A (Willett et al., 2001). Additionally putative inhibitor(s) in F3 HE-CEWAF might only inhibit when the F3 HE-CEWAF concentration is very high.

4.4.5. General Conclusions

In this Chapter, the most potent HFO 7102 fraction, F3, was used to study the process of CYP1A induction by HFO in more detail. As noted in Chapter 3, F3 contained inducer(s) of EROD activity and CYP1A protein. As F3 exposure times were increased, higher F3 concentrations were needed to induce EROD activity. One possible explanation is that with time RTL-W1 metabolizes the destruction of EROD inducer(s). With short exposures, the inducer(s) have yet to be metabolized so EROD induction occurs with a relatively low dose. With longer exposure times, more inducer(s) would be destroyed and higher concentrations of F3 would be needed to induce EROD. Possibly the induced CYP1A catalyzes the destruction of the inducer(s). The destruction of inducer(s) is further suggested by experiments where CYP1A protein levels were followed with time after exposure to F3 had been ended. CYP1A protein levels declined. However, in addition to CYP1A inducer(s), F3 appeared to contain inhibitors of CYP1A catalytic activity. This is suggested by the responses to high F3 concentrations: little EROD activity was detected but the level of CYP1A protein was high. This was further supported by experiments where RTL-W1 were exposed to F3 and TCDD together: CYP1A protein was induced but not EROD activity. Therefore, F3 contains both inducer(s) of CYP1A protein and inhibitor(s) of CYP1A catalytic activity.

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Appendix A Chemical analysis HFO 7102 and its fractions

Table A.1. Weight distribution of each fractions in HFO 7102

Fraction*	Mass (g)	% of Whole Oil
F1	0	0
F2	250.3	6.8
F3	937.1	25.4
F4	2426.4	65.7
Total	3613.8	97.9

All the data in this table was provided by Bornstein (Master thesis, 2012)
* Each fraction was separated based on the boiling temperature of n-alkanes with different carbon numbers; F1 were failed to collecting is because the IBP of HFO 7102 is higher than 174 °C

Table A.2. HFO 7102 and its fractions chemical composition from GC-MS

Compound	Whole Oil ($\mu g \cdot g^{-1}$)	Fraction 2 ($\mu g \cdot g^{-1}$)	Fraction 3 ($\mu g \cdot g^{-1}$)	Fraction 4 ($\mu g \cdot g^{-1}$)
$\sum$Alkanes	23,495	51,208	83,366	8,721
$\sum$ Methylated PAHs	23,861	47,135	79,205	5,909
$\sum$ Unsubstituted PAHs	2,150	5,384	5,923	602
Total Petroleum hydrocarbons	49,506	103,727	168,494	15,472

The whole oil was separated into Fraction 2, 3 and 4 by low temperature vacuum distillation. The whole oil and Fraction 2-4 were characterized by GC-MS. All the raw data was collected and analysed by Bornstein (Master Thesis, 2012)

Table.A.3. Distributions of PAHs and alkylated PAHs in HFO and its fractions

Number of Rings	Whole Oil (%)	F2 (%)	F3 (%)	F4 (%)
$\sum$ 2 Ring PAH [a]	26.0	56.1	21.8	3.6
$\sum$ 3 Ring PAH [a]	23.5	32.3	32.1	2.5
$\sum$ 4 Ring PAH [a]	49.3	11.4	45.8	87.8
$\sum$ 5-6 Ring PAH [a]	1.2	0.1	0.4	6.1
Total PAH ($\mu g \cdot g^{-1}$) [b]	**26012**	**52519**	**85128**	**6511**

PAH chemical distribution for the whole oil and its fractions was generated from GC-MS; all the raw data was collected and analysed by Bornstein (Master Thesis, 2012);
[a] PAH concentration with each ring numbers was expressed as the percentage of total PAH in corresponding oil sample
[b] The total PAH concentration in each oil sample was expressed as $\mu g \cdot g^{-1}$

Table A.4. Chemical Compositions of F3, F3-1 and F3-2 by HPLC-UV-Vis

Compound	Fraction		
	F3 ($\mu g \cdot mL^{-1}$)	F3-1 ($\mu g \cdot mL^{-1}$)	F3-2 ($\mu g \cdot mL^{-1}$)
Total Alkanes	83,368	22,351	271,303
Total Methylated PAHs	79,205	85,672	181
Total Unsubstituted PAHs	5,923	6,399	0
Total PAHs	**85,128**	**92,095**	**181**

F3, F3-1 and F3-2 were extracted by open column chromatography and characterized by HPLC-UV-Vis ; all the raw data was collected and analysed by Bornstein (Master Thesis, 2012)
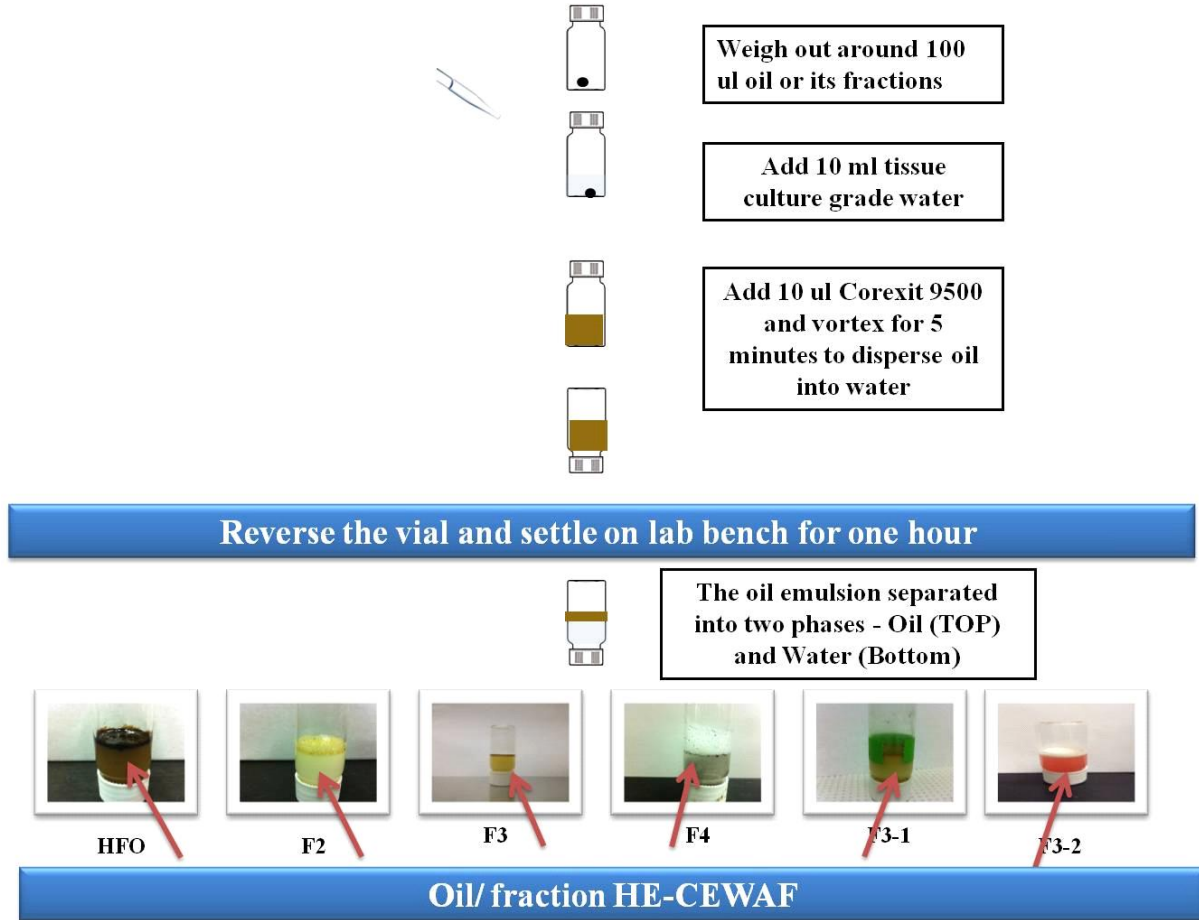
Appendix B HE-CEWAF Preparations

Weigh out around 100 ul oil or its fractions

Add 10 ml tissue culture grade water

Add 10 ul Corexit 9500 and vortex for 5 minutes to disperse oil into water

Reverse the vial and settle on lab bench for one hour

The oil emulsion separated into two phases - Oil (TOP) and Water (Bottom)

HFO   F2   F3   F4   F3-1   F3-2

Oil/ fraction HE-CEWAF

Figure B.1.Flowchart for oil/fraction HE-CEWAF preparation. *The key steps for making HE-CEWAF were outlined in each text box. The red arrow is pointing the HE-CEWAF portion of each oil or fraction. Specially, the pink colour of F3-2 is the color of culture medium instead of HE-CEWAFs*

Table B.1 Representive HE-CEWAF stock concentration of each oil sample

	HFO 7102	F2	F3	F4	F3-1	F3-2*
Oil volume (ul)	~100	~100	~100	~100	~100	~300
Oil mass (mg)	126.8	90.7	134.7	128.0	116.4	245.0
Corexit (ul)	10	10	10	10	10	N/A
Tissue culture water (ul)	10,000	10,000	10,000	10,000	10,000	10,000 (L-15/FBS)
HE-CEWAFs conc (mg/ml)	12.7	9.1	13.5	12.8	11.6	24.5
Corexit conc in HECEWAFs (% v/v)	0.1	0.1	0.1	0.1	0.1	N/A
oil to water ratio	1:100	1:100	1:100	1:100	1:100	N/A
Dispersant to oil ratio	1:10	1:10	1:10	1:10	1:10	N/A

*F3-2 was diluted into cell culture medium directly instead of tissue grade water; No Corexit was used to disperse F3-2 into water
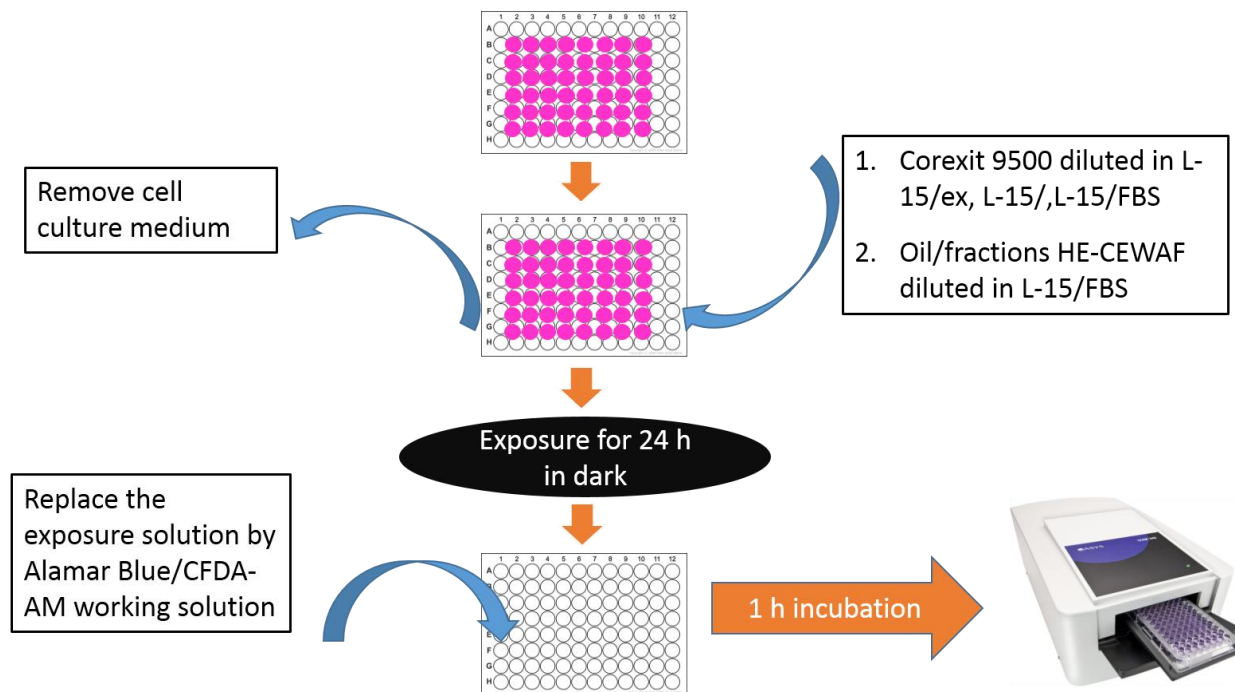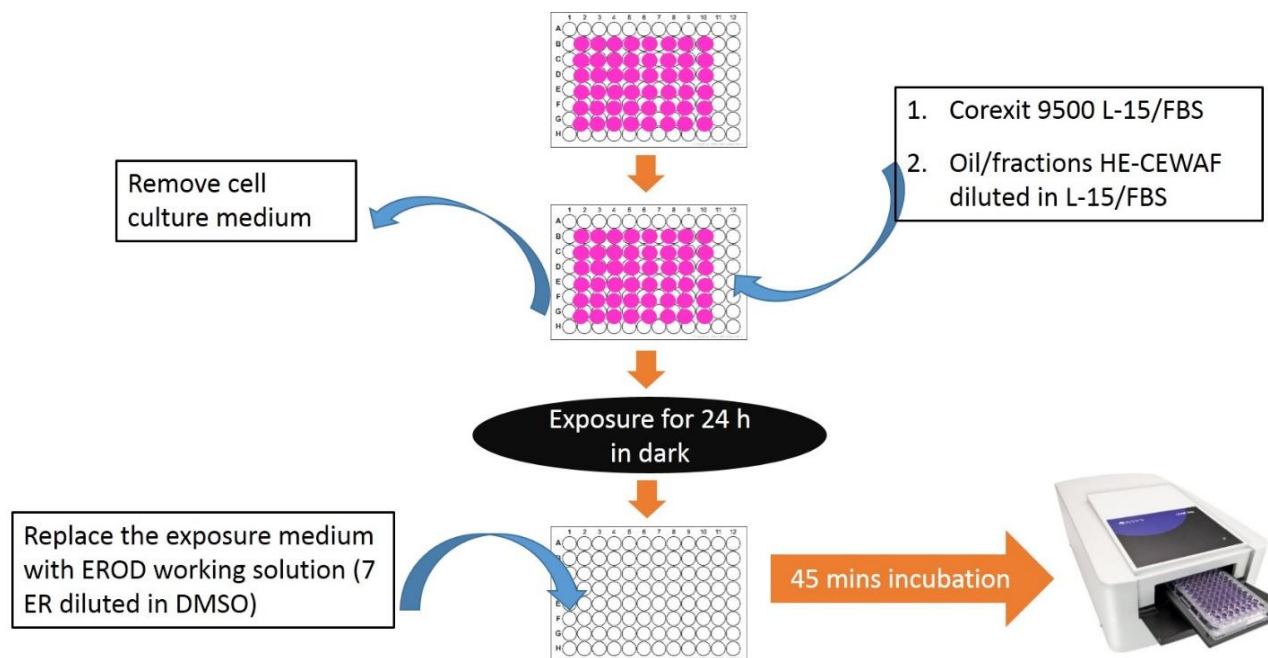
Appendix C Schematic flowchart for methodology

Figure C.1. Schematic flowchart of Cytotoxicity measurement in RTL-W1.

FigureC.2. Schematic flowchart of measuring EROD activity in RTL-W1.

1. Corexit 9500 L-15/FBS
2. Oil/fractions HE-CEWAF diluted in L-15/FBS

Remove cell culture medium

Exposure for 24 h in dark

Collect cell lysates

Protein separation

Protein detection

1° antibody: Monoclonal mouse antifish
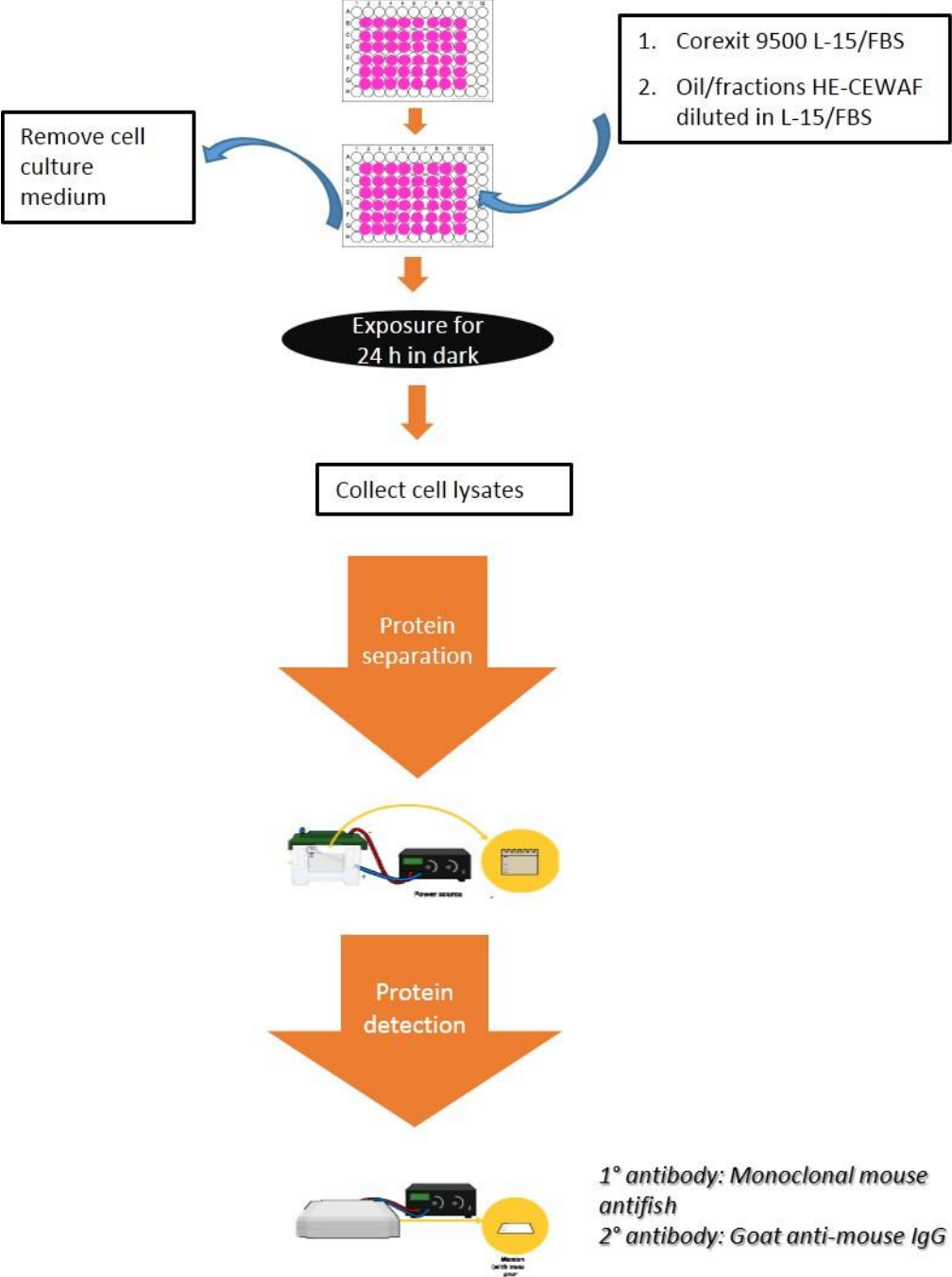2° antibody: Goat anti-mouse IgG

Figure C.3 Schematic flowchart for measuring the CYP1A polypeptide level by Western blot in RTL-W1.
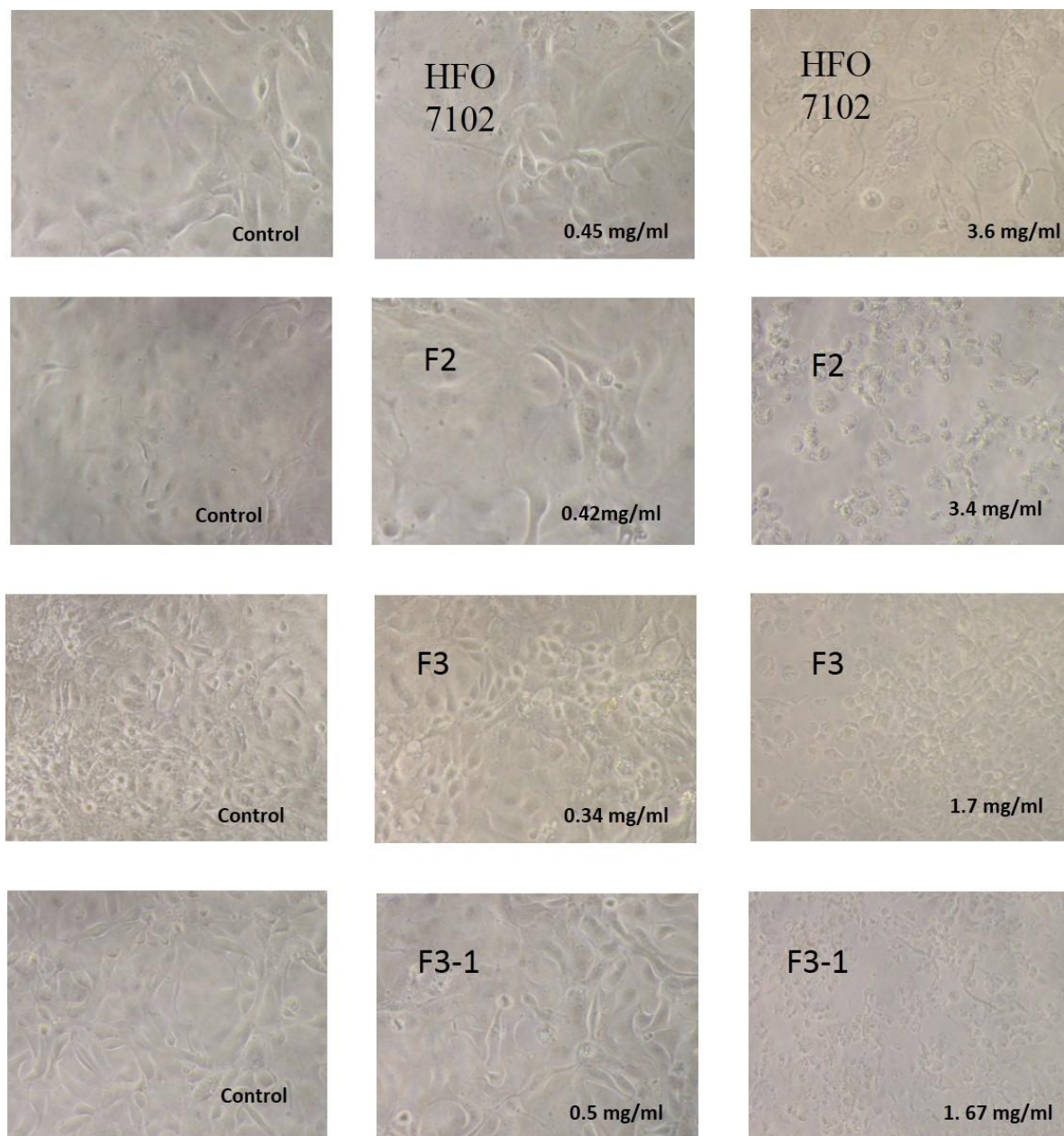
Appendix D. Morphology of RTL-W1

Figure D.1. Morphology of RTL-W1 exposed to different concentration of oil/fractions HE-CEWAF. *RTL-W1 was exposed to HFO or its fractions HE-CEWAF for 24 h in dark. At the end of exposure, cells in each well was examined under contrast microscope. Each picture represents one of the 6 repeated wells. The exposure chemical and concentration was label in each photo.*