How toxic is oil?

Investigating specific receptor-mediated toxic effects of crude and refined oils

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Front cover: Alaska, aerial view of Rocky Point in Kenai Fjords. Coast line damaged with oil from Exxon Valdez, © Ken Graham/AccentAlaska.com
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Hoe toxisch is olie? Onderzoek naar specifieke receptor gemedieerde toxische effecten van ruwe en geraffineerde olie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G. J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 7 oktober 2011 des ochtends te 10.30 uur

door

Cozmina Maria Vrabie

geboren op 9 november 1979 te Galati, Roemenie

Promotoren: Prof.dr. M. van der Berg

Prof.dr. A. J. Murk

Co-promotor: Dr. M. T. O. Jonker

To my parents, Maria and Mircea

"The Earth does not belong to us, we belong to the Earth. Whatever befalls the Earth befalls the sons and daughters of the Earth. We did not weave the web of life, we are merely strands in it. Whatever we do to the web we do to ourselves."

Chief Seattle, 1854

Contents

Chapter 1

General introduction

Environmental oil pollution

Oil pollution may occur in basically all the environmental compartments: sediment, soil, water and air. The sources of this pollution can be natural (seepages) and anthropogenic (leakages and accidents) [1]. Kvenvolden and Cooper (2003) have estimated that about 47% of crude oil currently entering the marine environment is from natural seeps with an average of 600,000 metric tons annually [2].

All the human activities related to the crude oil life cycle, such as extraction, transportation and storage, as well the uses of refined crude oil products can result in anthropogenic pollution. This can be either unintentional due to failing extraction (land and offshore) and transport equipment (pipelines and tankers), dry docking, marine terminals decommissioning of ships, leakages from garages, gas stations, bunker ships, yacht harbors, runoff from rail roads and highways, or due to intentional dumping of waste oil from ships or cars [1]. Media and public attention, however, mostly focuses on accidental oil spill events, especially at sea. Between 1978 and 1995 the annual volume of accidental spills varied between 960 and 57 million liters [3]. In 1989, the Exxon Valdez accidentally spilled 42 million liters of Alaskan North Slope crude oil into Prince William Sound. Approximately 2000 km of pristine shoreline was contaminated resulting in one of the worst ecological disasters in the US history [4]. In April 2010, the explosion of the Deepwater Horizon offshore oil platform in the Golf of Mexico resulted in the largest oil spill ever. Governmental and non- governmental agencies estimated that approximately 5 million barrels (895 million liters) of oil was spilled and polluting an area of about 75,000 km^2 [5].

As a result of these spills, the concentration of dissolved petroleum hydrocarbons in water can vary from high concentrations at the point of release to gradually lower concentrations as the oil is transported away from the source. Concentration of 0.5 to 10 mg/L of dispersed and dissolved oil near the point of release for IXTOC-I (i.e. offshore rig in the Campeche Bay, Mexico) well blowout have been reported [1]. In case of the Deepwater Horizon spill, the US National Oceanic and Atmospheric Administration (NOAA) reported concentrations of 10 µg/L of semi-volatile petroleum hydrocarbons (TPHs) at depths of 1000 - 1300 m [6]. Other authors report concentrations of in the range of 50 mg/L to 1.5 mg/L after a spill with concentrations decreasing to background level after approximately 1 month [7]. The oil in the water column can either degrade or settle in the sediment. The background average concentrations reported in the Netherlands Continental Shelf are in range 10 µg/L [8].

Composition of oils

Crude oils were formed millions of years ago through trapping of microscopic plants and animals in sediments and rocks at the bottom of the sea [9]. Crude oils are complex mixtures of many thousands of predominantly organic and some inorganic substances. The main fraction is made up of hydrocarbons (chemicals containing only C and H atoms),

followed by the hydrocarbon substances containing nitrogen (N), sulfur (S) and oxygen (O), generally called NSO compounds. Examples of NSO compounds are the so-called asphaltenes and resins which are polar, polynuclear molecules (Figure 1.1). Asphaltenes are known for their high molecular weight [10,11]. The smallest fraction of the total mass of crude oil is formed by metals, such as nickel, vanadium, and sulfur [1]. Depending on the extraction well, the composition of crude oil may vary greatly [12].

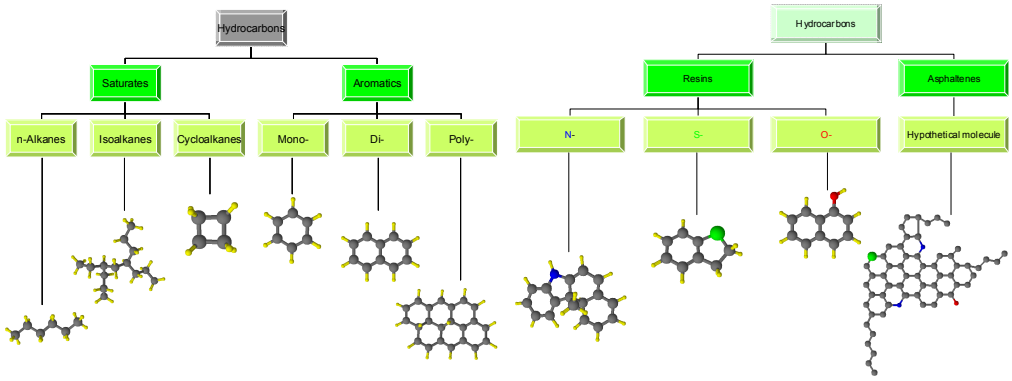

Figure 1.1. Example of hydrocarbons and non-hydrocarbons present in oil (adapted from Evers et al. 1997)

The petroleum industry classifies crude oils based on their geographical provenience (Arabian, Alaskan North Slope, North Sea, etc). This classification, however, is not used in case of a response to an oil spill, as it provides no information on the toxicity, physical state, and changes in the oil's composition upon release in the environment [13]. In oil spill responses the classifications light, medium, and heavy is used, based on the specific gravity of the oil [13]. In order to be able to classify crude oils according to their toxic effects, a classification on the chemical composition is often made into non-aromatic and aromatic classes [14]. The non-aromatics include n-alkanes (n-paraffins), branched alkanes (isoparaffins), cycloalkanes (cycloparaffins or naphthenes), and alkenes (olefins) [15]. The alkenes and aromatics can also be grouped as unsaturates, while the alkanes (aliphatics) are also referred to as saturates. Benzene, toluene, ethylene, and xylene (BTEX) are examples of monoaromatic compounds found in oils. Polycyclic aromatic hydrocarbons (PAHs) are having two or more fused rings and range from naphthalene to coronene [15].

Refining crude oil into products is a complex process involving steps such as separation of oil components by distillation, conversion of the distillation products into high valued fuels, and purification. In the first step of distillation, the crude oil is separated into different, light and heavy, unfinished products based on their boiling point and carbon number ranges. The lightest fraction, liquefied petroleum gas (LPG) will separate first, while

the heaviest fractions, such as bitumen will remain in the separation furnace. Next, in the conversion step, several processes (e.g. catalytical cracking, hydrocracking) are applied to the heavy hydrocarbons products resulted from the distillation step in order to obtain high value end products such as gasoline, jet and diesel fuels, lubricants, etc. Other processes in the conversion steps such as catalytical reforming are intended for rearranging paraffins into aromatic compounds that are further converted into for example, plastics. In the final step of purification, the main goal is the removal of sulfur from the final products [16]. Apart from their use as fuels, there is a vast and diverse market for products derived from the refining of crude oil. These products include ink, dishwashing liquids, deodorant, tires, CDs and DVDs [17], plastics, dyes, perfumes, soaps, insecticides, etc. [18].

Weathering of oil

Following the event of a spill on either land or water, the oil will undergo a series of physical, chemical and microbiological transformation processes, collectively referred to as weathering. Weathering not only affects the volume of the spilled oil, but also its chemical composition and resulting toxicity. The physical characteristics of crude oils and refined products will determine their movement in the environment, whereas for example volatility and solubility determine whether the petroleum hydrocarbons will partition into air, water and groundwater. Notably, the climate and as well the weather conditions at the place of spill site contribute to the speed of the weathering process. In case oil is spilled on land, the weathering processes will involve mainly evaporation, photooxidation, and degradation by soil microorganisms.

As soon as oil is spilled onto the water surface, it spreads out to form oil slicks on the surface of the water. The main mechanisms which govern the fate of an oil slick at sea are spreading, evaporation, dispersion, emulsification, sedimentation and biodegradation [19]. Fate modeling shows a gradual increase of the sediment concentration with a decrease of the concentration in the water column during several weeks [20].

Biodegradation and evaporation of low-molecular weight aliphatics and aromatic hydrocarbons will occur during the first days following a spill, resulting in an increase of the polar fractions (asphaltenes and resins). The polar fractions together with the high molecular weight hydrocarbons are more resistant to biodegradation. Their persistence makes them therefore interesting candidates for studying the long-term effects on ecosystems [21]. Nevertheless, the persistence of oils in the environment is highly dependent on the environmental, microbiological and geological conditions of the site [22]. Regarding this, there is a change in oil's ecotoxicity paradigm, as Peterson et al. (2003) stated that "oil degrades at varying rates depending on the environment, with subsurface sediments physically protected from disturbance, oxygenation, and photolysis, retaining contamination by only partially weathered oil for years" [4]. In case of the Exxon Valdez spill, subsurface oil was found to be present on Gulf of Alaska's beaches mid- and lower intertidal areas still 12 years later [23]. Short et al. (2007), reported that there was little change in the subsurface

oil even in aerated sediments, probably due to percolation of the viscous oil into porous sediments [24]. Moreover, these puddles of oil-polluted sediments are protected against natural dispersion by the large cobbles and boulders [25]. In case of spills occurring in tropical waters, the tendency would be to believe that the weathering process will be faster. However, Burns et al. (1993) reported that the medium-weight crude oil spilled on the Caribbean coast of Panama persisted up to 20 years due the slow release of the oil trapped in anoxic sediments [26]. In marsh sediments from West Falmouth, MA the additional high concentration of organic carbon contributed to long-term persistence of petroleum hydrocarbons [27,28]. One should therefore not generalize the fate and persistence of petroleum hydrocarbons in the environment. As seen from the above-mentioned studies, certain oil types can persist up to many decades, while in other cases the level of petroleum hydrocarbons (kerosene) can decrease to insignificant levels after 2 years [29].

Therefore, given the difference in the composition of the initial crude oil or refined product and the weathering rate in the environment, the resulting weathered oils will as well vary in their composition and thus toxicity [30].

Toxicity of oil

The effects of oil spills on wildlife habitats depends on the season in which the spill occurs, quantity and type of the oil spilled, environmental conditions, and sensitivity of the exposed species. Moreover, certain chemical parameters, such as volatility, solubility, octanol-water (K_{OW}) and soil or sediment-water (K_d) partition coefficient, and bioconcentration factor (BCF) will determine the components of oil remaining after a spill, what their environmental compartment movement will be and whether they will accumulate in biota. More importantly, the bioavailability of petroleum hydrocarbons for uptake into organisms is determinant for their ability to cause acute and chronic effects.

As already mentioned in this chapter, oil is a complex mixture and so are the adverse (e.g. smothering) and toxic effects (e.g. narcosis and specific chronic toxicities) on biota. All these effects will be briefly discussed below. Smothering occurs at very high concentrations of oils immediately after a spill. The adverse effects on organisms can range from effects on photosynthesis and respiration in green algae, to damaged photosynthesis due to fouling, prevention of shoot and leaf regeneration in coastal marsh plants, to mortality in invertebrates [31-35]. Birds and marine mammals may suffer from smothering with oil of feathers and fur, egg oiling, and ingestion food and water. For example, death by hypothermia and drowning are caused as a result to oiling of fur and feathers [4].

Acute toxicity occurs when an organism is still exposed to high concentrations of petroleum hydrocarbon, such as the organisms living in the water column just below a spill. This type of toxicity is called narcosis or baseline toxicity (acute non specific toxicity) and is assumed to be caused by inert chemicals through partitioning of, in this case, the petroleum hydrocarbons in biomembranes, thereby affecting their structure and functioning [36,37]. The properties and functionality of the lipid membrane will change due to the narcotics by

increasing the permeability and fluidity of membrane phospholipids (reviewed in [37]). The death will occur due the decreased activity of the organism and its reduced capacity to react to external stimuli [37]. There are two type of narcotic chemicals, type I nonpolar and type II polar. For type I inert chemicals, the lethality is assumed to be a function of the hydrophobicity and is expressed as log octanol-water partition coefficient (log K_{ow}) [38]. The type II chemicals deviate from this assumption and therefore in their cases the assumption is that toxicity is caused through a different mode of action [38].

Specific, chronic effects occur in organisms following the short or long term exposure to low concentrations of petroleum hydrocarbons. Ingestion of contaminated food, water and preening activities are some of the chronic exposure routes to petroleum hydrocarbons. The various sublethal effects reported in the literature include endocrine disruption in mussels, herring gulls and black guillemots, behavioral changes in amphipods, genotoxic effects in amphibians and cod fish, effects on embryonic and larval development in fish, embryotoxicity and effects on the reproduction on Mallard ducks, altered erythrocyte homeostasis, and hepatic metabolism and adrenal physiology in mink [35,39-48].

Recently, oil was classified in the category of contaminants of concern that persist in environment, bioaccumulate in biota and cause adverse effects [49]. Therefore, detailed insight in the mode of toxic action, identification of specific chemicals responsible for toxicity, and correlating their hydrophobicity to bioaccumulation can help to isolate the active chemicals, identify their biotransformation potential, and possible shed light on whether there is potential for biomagnification to top predators [50]. Preferably, the obtained knowledge should be included in future risk assessment of oils.

Intermezzo – endocrine disruptors and mechanisms of action

Worldwide regulatory bodies such as the US Environmental Protection Agency (EPA), the Organization of Economic Cooperation and Development (OECD), and the European Chemical Industry Council (CEFIC) have stimulated the development of fast *in vitro* alternatives to toxicity testing. These newly developed assays also made possible the fast screening of the toxicity of different oil types and the identification of the potential modes of action. So far, investigations using these assays have demonstrated that apart from genotoxicity several crude oils, refined products and their corresponding fractions have dioxin-like, (anti)estrogenic and (anti) androgenic effects in *in vitro* cell- and yeast-based assays [51-58].

Many man-made chemicals have been shown to interact with the steroid system and in the recent years evidence suggests that crude oils, refined petroleum products and offshore produced water are as well important sources of estrogens [40,59]. Arguably, the concern is that oils will contribute to the existing pool of endocrine disruptors and thereby causing feminizing effects and altering the reproduction in wildlife species. Given that oils are such complex mixtures, the exposure of an organism does not occur with one compound at the time. Therefore, some questions arise on whether the chemicals are going to influence

each other's bioavailability and toxic effects. Given the high mass of products formed during the refining process of crude oil, the oil and gas industry is the largest source of dioxins [60]. The dioxins can bioaccumulate and bioconcentrate in organisms (reviewed in [61]) such as fish and crab [62], affect reproduction in cormorants and vitamin A and thyroid hormone levels in otters [63,64]. Although the exact mechanism of toxicity is controversial, the evidence so far suggests that it is mediated via the aryl hydrocarbon receptor (AhR) [65]. The receptor is widely distributed among a variety of mammalian and non-mammalian species (reviewed in [65]) and also induced by polycyclic aromatic hydrocarbons (PAHs). Accordingly, several phase I and II biotransformation enzymes have been shown to be induced, such as the cytochrome P450s (CYP 1A1, CYP 1A2 and CYP1B1) and uridine-5-diphosphate glucuronyltransferase (UDPGT), glutathione-S-transferase P1 (GSTP1), uridinediphosphate glucuronosyl transferase 1A6 (UGT1A6), and NAD(P)H quinine oxidoreductase 1 (NQO1) [66,67]. PAHs have high affinity for the AhR and undergo biotransformation by the induced phase I an II enzymes which often increase their toxicity as genotoxic and mutagenic compounds [68,69]. High-ring PAHs tend to bioaccumulate in marine organisms because they are not easily metabolized and their toxicities are similar to those of dioxins in early life stages of zebrafish upon continuous exposure to PAHs [70,71]. Given that the evidence so far indicates the possible endocrine disruptive properties of oils and the existence of dioxins found in the environment near oil refineries, in depth and systematic research by applying *in vitro* assays into these specific effects of oils can perhaps help in the improvement of environmental risk assessment of oils.

Environmental risk assessment of oils

As mentioned above, the exposure of organisms and environmental hazard and risk of petroleum hydrocarbons will among others, depend on certain physical and chemical properties. Traditionally, environmental risk assessment of chemicals is based on predicted no effect concentrations (PNEC) as obtained from ecotoxicological tests. In the past, this parameter for oil was based on the whole product. This approach, however, does not take account that the numerous ingredients of oils have different persistence, hydrophobicities, etc. Without knowing more about the hazard of the most relevant toxic ingredients of the oils the risk assessment only will have limited validity.

Upon the initiative of the Conservation of Clean Water and Air in Europe (CONCAWE) oil companies' members therefore the hydrocarbon block (HB) model was developed. The principle of this model is based on separating the complex oil mixture into blocks containing chemicals with similar structures, physical and chemical properties, and thus arguably with the same environmental fate and hazard properties [72]. The advantages of the model are in the possibilities for making a species sensitivity distribution and for environmental risk evaluation, where a predicted environmental concentration (PEC) and a PNEC can be derived for each HB. In the end, the environmental risk of the complete oil mixture is calculated by the summation of individual HB PEC/PNEC ratios. The prediction of PNEC is based on the

non-polar target lipid model (TLM) [36,73]. The assumption of the model is that the chemicals are passively taken up into organism's lipids, their effects will be additive and for different compounds, the internal concentrations (molar basis) in the lipids at which narcosis occurs are equal [14,74-76]. The narcotic potency of petroleum hydrocarbons is assumed to be a function of the hydrophobicity and is expressed as log Kow [38]. Compounds with a log Kow > 5.6 are assumed to be non-toxic to organisms given their reduced bioavailability [77]. Within the TLM, the toxic unit concept (TU) can be used to account for differences in the general toxicities of individual compounds from the complex mixture [78]. The TUs are calculated based on the measured concentration of a chemical in the water divided by the median lethal concentration (LC50) of the same compound in the same medium. The estimated TU of the mixture are based on the summation of the individual calculated TUs [36]. This model serves as a basis for deriving the basic environmental risk limits (ERLs) for different compartments such as air, water, soil and sediment and further serve as advisory values for setting the environmental quality standards (EQSs) [79].

Recently, McGrath and DiToro (2009) took the TLM model one step further by validating it for the prediction of both acute and chronic, sub-lethal effects of water-soluble monocyclic aromatic hydrocarbons (MAHs) and those of PAHs [80]. This model is an important advancement in the risk assessment of oils as it stresses the need of accounting for not only non-specific but as well specific toxicities of petroleum hydrocarbons.

Oil is one of the most important sources of pollutants and proper hazard evaluation and identification is therefore necessary. Given the increased public and regulatory agencies awareness on the dioxin-like and endocrine disruptive properties of environmental pollutants and the increasing body of evidence for sub-lethal effect in wildlife chronically exposed to petroleum hydrocarbons it is essential that further models of petroleum hydrocarbons account (if possible) for these specific effects. The outcome can be used to decide whether clean-ups of oil spills and remediation of oil-polluted soils and sediments are needed.

Aim of the current thesis

Although some evidence exists on Aryl hydrocarbon receptor (AhR) activation and endocrine disruption properties of oil, the information is fragmentary, mostly with one type of oil that has been tested in only one particular assay. Most importantly, the capacity of petroleum hydrocarbons to outstand biotransformation and bioaccumulate in biota had not been thoroughly investigated. This information can be of importance when performing the environmental risk assessment of oils. Therefore, this thesis aimed to investigate possible AhR activation effects and interactions with hormonal system of environmentally relevant oils with very different natures (i.e. crude oils and refined petroleum products) in a series of *in vitro* bioassays. Next, the aim was to identify the chemical classes responsible for the effects by applying an effect directed analysis (EDA) and determine the bioaccumulation potential in biota based on the hydrophobicity.

Outline of the thesis

In order to meet the aims of this thesis, several studies have been conducted. The cytotoxicy and AhR agonistic activities of 11 crude oil and refined petroleum products were determined in the *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and dioxin-receptor mediated luciferase reported gene assay (DR-Luc) for stable (dioxin-like) and labile (PAH like) compounds in **Chapter 2.** Furthermore, investigation of possible antagonistic and additive effects was done by exposing the cells to different concentrations of oils in combination with the DR-Luc assay's positive standards benzo[*a*]pyrene (BaP) for the 6 h exposure and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for the 24 h exposure.

In Chapter 3, estrogenic and androgenic effects of the oils were studied in a recombinant yeast stably transfected with either human estrogen receptor alpha (ERα), beta (ERβ), or androgen receptor (AR), and expressing yeast enhanced green fluorescent protein. Furthermore, additive, antagonistic, and synergistic effects were studied in the assays by co-exposing the yeast to a fixed concentration of standard 17-β estradiol or testosterone (E2 or T) and increasing concentrations of oils. Mechanistic studies were performed in order to investigate whether the observed effects were receptor-mediated and therefore the yeast was co-exposed to the synthetic inhibitors ICI 182,780 (ERβ assay) or flutamide (AR assay), a fixed concentration of standard, and various concentrations of oil.

Further investigation and confirmation of the possible endocrine disruptive properties of oils that can occur in mammals was the subject of **Chapter 4**. By employing recombinant mammalian cells stably transfected with the human estrogen receptor alpha (ERα) or beta (ERβ), and expressing the luciferase protein (i.e., ERα-U2OS-Luc and ERβ-U2OS-Luc assay), the (anti)estrogenicity of oils was studied. More complex biological effects of oils such as cell proliferation and gene (pS2) expression were investigated in the estrogen-responsive human breast carcinoma cell line (MCF-7).

In Chapter 5, the bioaccumulation potential in the aquatic worms (*Lumbricullus variegatus*) of AhR agonists from oils was determined. In order to assess the bioaccumulation potential of oil and the possible induction of a chronic effect, the aquatic worms were exposed for 4 weeks to oil-spiked sediments, followed by their extraction, and application of the extract in the chemically-activated fluorescent gene expression cell bioassay (CAFLUX). Next, a separation of the oils into the so-called SARA (saturates, aliphatics, aromatics and resins) fractionations was performed. The fractions were tested in the DR-Luc assay and the active oil fractions were identified. The active fractions were sub-fractionated by reversed-phase HPLC into 9 sub-fractions corresponding to different Kow ranges, therefore indicative of different bioaccumulation potential. The sub-fractions were tested again in DR-Luc (6 and 24 hrs) and the active ones were isolated for further chemical characterization. This last step was performed by the means of gas chromatography with flame ionization detection (GC-FID) and mass spectrometry (GC-MS).

In **Chapter 6** the results of the previous chapters are summarized and discussed, and general conclusions are drawn.

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Chapter 2

Specific in vitro toxicity of crude and refined petroleum products: 1. Aryl hydrocarbon receptor-mediated responses

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Abstract

The present study is the first in a series reporting on in vitro toxic potencies of oils. The objective was to determine whether 11 crude oils and refined products activate the aryl hydrocarbon receptor (AhR) in a dioxin receptor-mediated luciferase assay. Cells were exposed for 6 and 24 h to different oil concentrations to screen for polycyclic aromatic hydrocarbon-like or dioxin-like activity. Moreover, cytotoxicity of the oils was determined using rat hepatoma cells. Except for one crude oil, none of the oils appeared cytotoxic up to 100 mg/L, but all oils activated the AhR. Strong AhR induction was observed for most oils after 6 h, and responses decreased after 24 h, indicating the presence of metabolizable agonists. However, several oils still caused high responses after 24 h, also demonstrating the presence of persistent agonists. The potencies (calculated based on comparisons of concentrations at which 50% of the maximal effect was observed) of oils were found to be approximately 40 to 10^6 times lower than the potency of the assay's standards benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. However, considering that oils contain thousands of chemicals, the potencies of petrochemical agonists may be very high. Among the most potent oils were bunker and crude oils. Induction up to 200% as compared to the maximum induction caused by benzo[*a*]pyrene was observed for these oils. Such supermaximal responses suggest mixture effects that may not be receptor-mediated. Experiments in which oils were tested in combination with the standards demonstrated that oils acted via an antagonistic or additive mode. The results of the present study may help improve risk assessment of petroleum products and judge the necessity or priority of oil spill cleanup activities.

Introduction

Oil is an extremely complex mixture of petroleum hydrocarbons, and its chemical composition can vary widely with its source [1]. Even after refining crude oil, the resulting products can consist of thousands of individual chemicals, such as aliphatic compounds (straight and branched alkanes), aromatic compounds (benzene derivates, polycyclic aromatic hydrocarbons [PAHs]), resins, and even inorganic compounds (vanadium and sulfur) [2,3]. Oil can end up in the environment via natural seeps or anthropogenic activities, including accidental spills from ships, (offshore) oil production, and car repair or dumping [4]. Risks associated with oil contamination of the environment are generally assessed only on the basis of the mixture's narcotic properties [5]. In other words, it is assumed that the toxicity of oil is solely caused by a disturbance of biomembrane structure or functioning because of partitioning of petroleum hydrocarbons into the membranes, a mechanism referred to as nonspecific or baseline toxicity [5,6]. Nevertheless, it is well-known that oils may contain compounds that can bring about specific toxic responses. Important examples are PAHs, which have been identified in many oils [7,8] and are known to cause mutagenic

and carcinogenic effects [9,10]. Accordingly, these chemicals might explain the genotoxic effects of oils often observed in in vitro and in vivo studies [11,12]. The same class of chemicals has also been suggested to cause direct and indirect (anti)estrogenic [13-16] and antiandrogenic effects [17] of oils in vitro. Furthermore, dioxin-like aryl hydrocarbon receptor (AhR)-mediated effects have been reported [18,19].

Specific effects of oils have not yet been studied in a systematic way, and the causative (classes of) compounds have not been identified. At present, little information on specific effects is only available for a select number and type of oils. Still, understanding the potential toxic effects of oils is necessary for proper environmental and human risk assessment of the complex mixtures.

The present study is the first in a series reporting on a systematic investigation of specific, receptor-mediated toxic effects of oils. Effects of a diverse set of 11 oils were studied by using several different in vitro assays, focusing on binding to estrogen (alpha and beta), androgen, thyroid, or the AhR. Also, we attempted to identify the groups of compounds responsible for the observed effects by fractionating the oils. The oils were chosen to cover environmentally relevant, real-world (not commercially available) crudes and refines, fuel and lubricating oils, and light and heavy products. The objective of the present study was to determine the cytotoxic potential of the oils, as well as their ability to interact with the AhR. Cytotoxicity was quantified on basis of the decrease in cells' metabolic activity by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [20]. Interaction with the AhR was studied by dosing the oils in the so-called dioxin receptor-mediated luciferase reporter gene assay (DR-Luc assay) [21-23]. To be able to distinguish between easily biodegradable compounds (e.g., PAHs) that can cause a response in this assay after only 6 h but have degraded after 24 h and more persistent compounds that still induce the AhR after 24 h [24,25], exposure duration lasted for both 6 and 24 h. Furthermore, to investigate possible antagonism and non-receptor-mediated mixture effects that would not be observed if the oils were tested alone, the oils were tested in combination with the assay's positive standards benzo[*a*]pyrene (B*a*P) for the 6-h exposure and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for the 24-h exposure.

Materials and Methods

Chemicals

All chemicals were at least 98% pure, unless stated otherwise. Solvents used were ethanol (LiChrosolv grade; Merck), toluene (Spectranal grade; Riedel-de Haën), dimethylsulfoxide ([DMSO]; spectrophotometric grade; Acros Organics), and hexane and acetone (pestiscan grade; Labscan). Both B*a*P and TCDD were internally validated standards from the Section Toxicology, Wageningen University. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Invitrogen. *trans*-1,2-Diaminocyclohexane-*N*,*N*,*N*',*N*'-tetraacetic acid

monohydrate (CDTA) was obtained from Fluka. Tricine was purchased from Sigma-Aldrich. Magnesium carbonate was obtained from Sigma-Aldrich. Sodium hydroxide, ethylenedinitrotetraacetic acid (EDTA·$2H_2O$), magnesium sulfate ($MgSO_4$·$7H_2O$), and 1,4-dithiothreitol were obtained from Merck. d-Luciferin was purchased from Duchefa. Adenosine triphosphate was obtained from Roche.

Oils

Eleven oils were tested in the present study. There were four crude oils and seven refined petroleum products, of which two were bunker oils. An overview of the names of the oils, their sources and applications, and the abbreviations used throughout the article is provided in Table 2.1.

Table 2.1*:* Refined petroleum products and crude oils used in the present study

Name	Abbreviation	Source or supplier	Application
Refined products			
Gasoline	GO	BP gas station, Wageningen, The Netherlands	Commercial fuel in cars (Euro 95)
Kerosene	KS	Air Force base, Soesterberg, The Netherlands	Fuel in military helicopters
Distillate Marine grade A	DMA	Gulf Oil, Nigtevecht, The Netherlands	Gasoil in midsized to larger ships, such as coasters, large cutters, and inland vessels
Engine oil	EO	BP gas station, Wageningen	Commercial lubricant for cars (Visco 2000)
Bilge oil	BIL	Oil layer scooped from a bilge water collection depot at a yacht basin, Wageningen	None; waste product
Bunker 180 (Heavy nautical fuel oil)	B180	Atlantic BV oil company, Pernis, The Netherlands	Fuel in large ships
Bunker 500 (Heavy nautical fuel oil)	B500	Atlantic BV oil company, Pernis	Fuel in large ships
Crude oils			
Arabian crude	AC	Shell refinery, Pernis; extracted in Saudi Arabia	Used for refining into petroleum products
Romanian crude	RC	Extracted at the Tintea oil field, Ploiesti, Romania	Used for refining into petroleum products
Oseberg crude	OC	Shell refinery, Pernis; extracted at the Oseberg area, Norway	Used for refining into petroleum products
Hollmix crude	HMC	Shell refinery Pernis; offshore product from the North Sea	Used for refining into petroleum products

Dissolution and dilution of the oils

All oils needed to be dissolved in an appropriate carrier solvent before they could be dosed in the in vitro assays (usually DMSO). Carrier solvent requirements obviously are the ability to dissolve the oils and miscibility with water. These (apparently conflicting) requisites have been complied with before by dissolving oils directly in methanol [18], DMSO [15,16], or ethanol [11,14,17] or by mixing oils initially diluted in hexane with methanol [18]. However, when preparing oils for dosing in the present study, none of the oils except for gasoline (GO), kerosene (KS), and bilge (BIL) oil dissolved at sufficiently high concentrations in DMSO, methanol, or ethanol. Also, the bunker and several crude oils did not fully dissolve in hexane or heptane. Therefore, all oils were dissolved 1:10 in toluene (about 100 g/L), a solvent

capable of dissolving all oils studied. Subsequently, these toluene stocks were diluted 1:5 and 1:20 in ethanol (in duplicate), yielding oil concentrations of 20 and 5 g/L, respectively. Using this solvent bridge, all oils could be dissolved in a water-miscible solvent. The final test dilutions were then prepared by diluting the 5 g/L of ethanol solution in nine successive steps of 1:10 down to a concentration of 50 ng of oil/L of ethanol. All the dilutions were prepared in 1.8-ml amber glass vials with polypropylene screw caps (Grace Alltech Associates). All glassware was rinsed with acetone and toluene before use.

Removal of sulfur

To remove elemental sulfur, approximately 200 mg of hexane-washed copper powder (>230 mesh; Merck) was added to each duplicate of the 1:5 and 1:20 oil dilutions in ethanol and the vials were sonicated for 30 min. The final test concentration series was then prepared from the resulting solutions as mentioned earlier.

Cytotoxicity assay

The MTT assay was applied to study the cytotoxicity of oils to rat hepatoma (H4IIE.luc) cells. The endpoint of the MTT assay is the decrease in the cells' metabolic activity. Experiments were performed according to the MTT protocol [20] with several adaptations. We plated 200 µl of cells suspension, at a concentration of 3×10^5 cells/ml, in the center 60 wells of a 96-well plate (Greiner Bio-one). To prevent evaporation of medium from the center wells, the outer rows of the plate were filled with 200 µl of Hank's Balanced Salt Solution (Gibco-Invitrogen). After 24 h, during which cells were allowed to attach, 1 µl of oil solution (treated either with or without copper powder) was directly added to 200 µl of 10% fetal calf serum (Gibco-Invitrogen) containing growth medium of the cells, resulting in final concentrations of 25 or 100 mg of oil/L. In addition, on each plate the following controls were included: 4% (v/v) DMSO (positive control), 0.5% (v/v) ethanol, and medium only (negative controls). Following the dosing, the plates were shaken at 400 rpm on an orbital shaker (Incubator 1000, Hieroglyph Instruments) for 5 min. After 21 h of incubation in a humidified atmosphere at 37^0C and 5% CO_2, the yellow MTT reagent was added to a final concentration of 0.5 mg/ml. To allow the viable cells to metabolize the dye into purple formazan crystals, the plates were then placed back in the incubator for an additional 3 h. Next, the medium was removed, 100 µl of DMSO was added, and the plates were placed on a shaker for 5 min to dissolve the formazan crystals. Subsequently, the absorbance was measured at 562 nm (color of the formazan) and at 620 nm (cell debris and other nonspecific absorbance) using a Multiskan Spectrum (Thermo Fisher Scientific) [26]. Pilot experiments had indicated that under the preceding experimental conditions the oils did not interfere with the absorbance measurements. Viability of the cells was finally expressed as percent formazan formation, relative to the maximum viability measured for the (0.5%) ethanol

solvent control. Both oil solutions and controls were tested sixfold, and the experiments were replicated twice. Percent standard deviations were 2 and 10% for the negative and positive controls, respectively.

DR-Luc assay

The DR-Luc assay was performed using H4IIE.luc cells, according to the method described by Murk et al. [23] and by applying some small modifications. In short, cells were grown in culture flasks in minimal essential medium (Gibco-Invitrogen), supplemented with 10% fetal calf serum at 37^0C and 5% CO_2. After the cell layer had reached 70 to 90% confluence, the cells were trypsinized and diluted. Then, 100 µl of the resulting suspension was plated into each of the 60 center wells of 96-well plates (Packard, ViewPlate^{TM}-96, white, PerkinElmer Life and Analytical Sciences). The cells were grown overnight until 100% confluence. Before the oil exposure experiments, pilot experiments were performed to determine the most appropriate exposure times. To this end, full dose-response curves of B*a*P and TCDD were measured after 6, 24, 48, and 72 h of exposure. Based on this initial test, 6 and 24 h were selected as exposure times (see *Results and Discussion* section). The exposure of cells to oil solutions only was done as follows: For the 6-h exposure experiments, just before exposure, 100 µl of preconditioned medium was added to the 100 µl of medium already being present in the wells. Preconditioned medium is medium in which cells have previously grown for 16 h, and it is used to avoid a high background signal induced by tryptophan products present in the fresh growth medium [24] that can also induce the AhR [27,28] and will therefore cause false-positive results. Tryptophan is metabolized by the cells within approximately 12 h of incubation. In case of 24-h exposure experiments, 100 µl of fresh growth medium was therefore added to the wells. Combination experiments were performed by dosing the cells with different concentrations of oils (0.025-100 mg/L) plus a fixed concentration of standard: 12,500 pM B*a*P (i.e., 3.2×10^{-3} mg/L; 80% effective concentration) for 6-h exposure experiments and 12.5 pM TCDD (i.e., 3.2×10^{-6} mg/L; 20% effective concentration) for 24-h exposure experiments. Note that the effective concentrations used here refer to concentrations for the induction. During previously performed pilot experiments, oils were first added to the media in a mixing plate, followed by dosing of this media to the cells. However, at high concentrations, the heavier oils were observed to form oil films on top of the medium in the mixing plate. To prevent loss of oils in the mixing stage, dosing of oils in the actual experiments was therefore performed by directly pipetting 1 µl of the oil dilutions into the 200 µl of medium in the wells. In addition to oil dilutions, each plate included control wells (medium only), solvent controls (0.5% ethanol for oils and 0.5% DMSO for standards), and calibration points. The calibration points used for 6-h exposures were 12,500 and 375×10^3 pM B*a*P, whereas for 24-h exposures, 12.5 and 375 pM TCDD were applied. In addition, full dose-response curves of B*a*P and TCDD were

prepared on separate plates. All exposures were performed in triplicate on the same plate, and the experiments were replicated twice.

After exposures, the medium was removed with a vacuum pump and the cells were washed with 0.5X phosphate buffer saline (without Ca^{2+} and Mg^{2+}; Gibco) at 200 µl per well. To allow swelling of the cells, 30 µl of hypotonic low-salt buffer (10 mM Tris, pH 7.8; 2 mM 1,4-dithiothreitol; and 2 mM CDTA) was added, and the plates were kept on ice for approximately 10 to 15 min. Subsequently, the plates were frozen for at least 30 min at -80°C to lyse the cells. Just before measuring, the plates were thawed on ice for 20 min and then placed for 10 min on a plate shaker (to reach room temperature). The measurements were performed with a luminometer, equipped with two injection pumps (Thermo Fisher Scientific). During the measurements, 100 µl of flashmix consisting of 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg\ (OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM $EDTA{\cdot}2H_2O$, 2 mM 1,4-dithiothreitol, 0.47 mM d-luciferin, and 5 mM adenosine triphosphate (pH 7.8) was added per well, followed by immediate addition of 50 µl of 0.2 M sodium hydroxide (which stops light production and therefore avoids quenching between neighboring wells), as described by Ter Veld et al. [29].

Data analysis

The responses induced by the oils and standards in the DR-Luc assay were analyzed using nonlinear regression in GraphPad 4 (Prism 4, GraphPad Software), and the standard curves were fit according to a sigmoid dose-response curve with variable slope. The signal of the solvent controls was set at 0 and the maximum signals of the standards at 100%. Hence, all data were presented as a percent response relative to the maximum effect of the standards.

The potency of the oils was determined on the basis of the 50% effective concentration (EC50) values, relative to that of the standard, and in addition expressed as B*a*P induction equivalents (IEQ_{BaP}) or TCDD induction equivalents (IEQ_{TCDD}). These values were calculated as described by Ziccardi et al. [18], by dividing the EC50 of B*a*P or TCDD by the EC50 of the oils (Eqn. 1 and 2). They represent the number of micrograms of B*a*P or TCDD equivalents present in 1 g of oil.

$$IEQ_{BaP} = \frac{EC_{50}BaP}{EC_{50}oil} \qquad (1)$$

$$IEQ_{TCDD} = \frac{EC_{50}TCDD}{EC_{50}oil} \qquad (2)$$

The limit of quantification, calculated as the luciferase induction elicited by the solvent plus three times the standard deviation, was interpolated in the dose-response curves from the oils to determine the lowest-effect concentrations (LECs) for the oils.

In case of the combination experiments, the measure for determining additivity, antagonism, or synergism was based on the descriptive method presented by Schroijen et al. [30]: measured responses for oils at 25 mg/L in combination with a standard were compared with the responses expected based on the summation of the individual response of the oils and that of the fixed concentration of the standard. Obviously, measured responses can be identical to, less than, or higher than the responses expected, reflecting additivity, antagonism, or synergism, respectively [30]. Measured and expected responses were considered statistically different (*t* test) if $p \leq 0.05$.

Data from the MTT assay were analyzed using a two-sample *t* test and assuming equal variances. Responses of oil-exposed cells were considered significantly different from the 0.5% ethanol control when $p \leq 0.05$.

Results and Discussion

Cytotoxic potential of oils

Reduced formazan formation by H4IIE.luc cells was only observed upon exposure to Romanian crude oil (RC) at a concentration of 100 mg/L (Fig. 2.1). However, exposure to 25 mg/L of this oil did not affect formazan formation (data not shown). All other oils tested were not cytotoxic to H4IIE.luc cells, neither at 100 mg/L (Fig. 2.1) nor at 25 mg/L (data not shown). It should be noted that even in the case of 100 mg of RC/L, microscopic inspection did not reveal any dead cells. Therefore, only the vitality of the cells may have been reduced by certain petroleum compounds at this concentration of RC. Still, the current data do not imply that oils cannot be cytotoxic, as Grant et al. [31] found that two aviation fuels were cytotoxic to H4IIE cells when exposed for 72 h to concentrations of 5 to 20 mg/L (50% inhibitory concentration of 12.6 ± 0.4 mg/L). The authors, however, used an exposure period that was three times longer, which can probably explain the difference with the results of the present study.

The current results did not show differences between the desulfurized and the untreated samples (data not shown). Despite the presumed presence of sulfur in several oils (personal communication with the suppliers), the sulfur concentrations apparently were not high enough to reach cytotoxic levels for the cells. Therefore, for further testing only untreated samples were used.

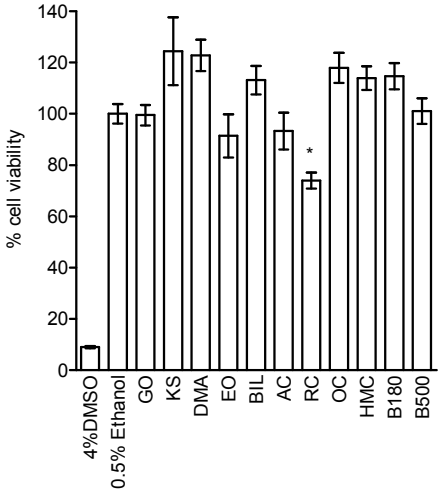

Figure 2.1: Viability of rat hepatoma (H4IIE.luc) cells exposed for 24 h to different oils at 100 mg/L (samples not treated with copper powder). Data (± standard deviation; *n* = 6) are expressed as formazan formation relative to solvent-exposed cells (0.5%); 4% dimethylsulfoxide (DMSO) represents the positive control. Significantly different from control (0.5% ethanol):* (p<0.05). Abbreviations are explained in Table 2.1.

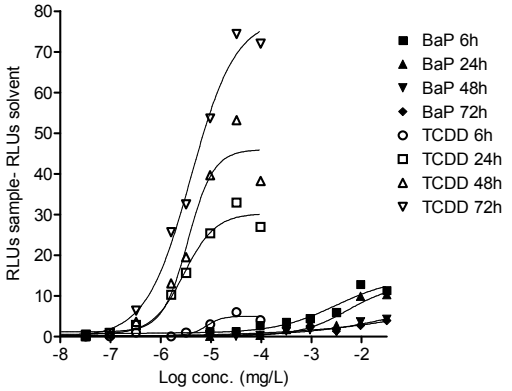

Figure 2.2: Aryl hydrocarbon receptor activation by benzo[*a*]pyrene (B*a*P) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rat hepatoma (H4IIE.luc) cells as a function of time. Results are expressed as relative light units (RLUs) of standards corrected for the background (RLUs sample – RLUs solvent). Cells were exposed for 6, 24, 48, and 72 h to B*a*P (0-0.0315 mg/L) or TCDD (0-9.6 x 10^{-5} mg/L). The error bars represent standard deviations (*n* = 3).

DR-Luc assay: Responses of BaP and TCDD over time

Due to the presence of cytochrome P450 (CYP) 1A activity in the H4IIE.luc cells, the concentration of easily metabolizable compounds, such as PAHs, decreases in time. This can be observed from the B*a*P and TCDD responses after 6, 24, 48, and 72 h (Fig. 2.2). At 6 h, TCDD induced the AhR only poorly, and the maximum induction by TCDD increased with time up to 72 h. For B*a*P, the highest AhR induction was measured after 6 h, and the inductions at 24, 48, and 72 h were progressively lower as compared to those at 6 h (Fig. 2.2). Although B*a*P was still inducing the AhR at the highest concentrations tested after 24 h, the response was approximately one-third of the TCDD response (based on the maximum responses). Therefore, 6 h was chosen as standard exposure time for the detection of B*a*P-like

compounds (i.e., easily metabolizable compounds), whereas 24 h was selected as exposure time for the detection of more persistent compounds. For the latter compounds, 24 h was preferred over the longer exposure times because of the possibility for faster screening. The preceding results are in accordance with those from Hamers et al. [24] and Machala et al.[25]. Machala et al. [25] also reported B*a*P induction to be higher after 6 h than after 24 h, whereas the reverse was observed for TCDD. It should be stressed that there are several versions of the DR-Luc assay available that differ in the cells and plasmids used. In all of them, TCDD is used as a positive control, but maximum induction is observed at different times, ranging from 4 h in case of the mouse hepatoma H1L1.1c2 cells to 24 h for the mouse hepatoma H1L6.1c2 cell line [32]. The exposure duration should therefore be tuned to the assay applied.

Table 2.2: Potency of oils relative to benzo[*a*]pyrene (B*a*P) following 6-h exposure in the DR-Luc assay

Standard/oil	LEC[a](mg/L)	Relative potency[b] (LEC)	EC50[c](mg/L)	IEQ_{BaP}[d](µg/g)	Max response[e] (%)	Conc.max[f] (mg/L)
B*a*P	9.8E-06	1	2.0E-04	1.0E+06	100 ± 2.5	0.009
Refined products						
Gasoline	1.9E-01	5.1E-05	3.874	5.2E+01	92 ± 5.3	25
Kerosene	9.3E+00	1.1E-06	NC[g]	NC[g]	46 ± 10	100
Distillate marine grade A	6.4E-03	1.5E-03	0.204	9.9E+02	118 ± 6.1	2.5
Engine oil	1.3E-01	7.4E-05	3.107	6.5E+01	83 ± 2.7	25
Bilge oil	2.8E-02	3.4E-04	1.758	1.2E+02	161 ± 8.7	25
Bunker 180	1.6E-03	6.2E-03	0.028	7.3E+03	164 ± 3.7	0.25
Bunker 500	2.2E-04	4.4E-02	0.008	2.7E+04	195 ± 9.9	25
Crude oils						
Arabian crude	1.0E-03	9.7E-03	0.071	2.9E+03	156 ± 3.9	2.5
Romanian crude	3.8E-03	2.5E-03	0.067	3.0E+03	150 ± 5.0	25
Oseberg crude	6.7E-03	1.5E-03	0.175	1.2E+03	156 ± 9.1	25
Hollmix crude	4.1E-03	2.4E-03	0.207	9.7E+02	151 ± 2.9	25

[a] Lowest-effect concentration (LEC); test concentration at which first effects are observed.
[b] Ratio of the dose of B*a*P and that of oil needed to achieve the LEC.
[c] Concentration at which 50% of maximum luciferase induction is reached.
[d] B*a*p induction equivalents; number of micrograms of B*a*P present in 1 g of oil.
[e] Percent luciferase induction (± standard deviation) calculated as the maximum response of oil relative to the maximum response for B*a*P.
[f] Concentration oil at which the maximum response is reached.
[g] NC = not calculated; maximum effect not reached.

DR-Luc responses induced by oils

Exposure of the H4IIE.luc cells to crude oils at concentrations of 0.025 to 100 mg/L for 6 h resulted in comparable responses. At 25 mg/L, RC, Oseberg crude oil (OC), and Hollmix crude oil (HMC) had responses amounting to approximately 150% in comparison to the maximum B*a*P induction. At 2.5 mg/L, Arabian crude oil (AC) had a response of approximately 150% in comparison to the maximum B*a*P induction (Table 2.2 and Fig. 2.3A). The concentrations at which first effects were observed were 0.025mg/L for AC and RC and

0.25 mg/L for OC and HMC (Fig. 2.3A). Exposure of cells for 6 h to refined products resulted in a variety of responses, ranging from approximately 50% (KS at 100 mg/L) to 200% (Bunker 500 [B500] at 25 mg/L) of the maximum B*a*P induction (Table 2.2 and Fig. 2.3B). Induction of the AhR by refined petroleum products has been reported in the literature before [18,19]. Ziccardi et al. [18] observed luciferase induction after a 3-h exposure duration in H1L1.1c2 cells for a series of refined products, including GO and KS at 50 mg/L. However, responses did not exceed the maximum responses observed for the assay's positive standard (TCDD), as observed in the present study. Jonker et al. [19] did observe responses for distillate marine grade A (DMA) oil in H4IIE.luc cells after 6-h exposure that exceeded the B*a*P maximum response (expressed as pM B*a*P equivalents).

After 24-h exposure in the present study, 25 mg of AC, OC, and RC per liter induced approximately 70% of the TCDD maximum response, whereas 100 mg HMC/L induced approximately 60% (Table 2.3 and Fig. 2.3C). The refined products induced between 3% (25 mg of KS/L) and approximately 80% (25 mg of Bunker 180 [B180]/L and 100 mg of B500/L) of the TCDD maximum (Table 2.3 and Fig. 2.3D). After 24-h exposures, responses were no longer observed to exceed the standard's maximum.

For all oils (except KS and BIL) and both exposure periods, luciferase induction was reduced at the highest concentration tested (100 mg/L) but no cytotoxicity was observed. Similarly, Ziccardi et al. [18] observed a decrease in luciferase induction in H1L1.1c2 cells exposed for 3 h to 50 mg of arctic diesel and aviation fuels/L and did not notice cytotoxicity at visual inspection. The authors hypothesized that the decrease might be explained either by toxicity caused by specific additives, inhibition of cellular processes, and/or the AhR induction pathway by petroleum compounds or oil additives, by increased interference or quenching of the light detection as a result of the darker color of the medium due to the high oil concentrations. Because the results of the present study demonstrate a decrease in the responses to both crudes and refines, and after both exposure times, the second and third explanation are most likely. One possible argument for the third explanation is that at the highest oil concentration a thin, floating layer of oil may be present. Such a layer might not (completely) be removed upon washing the cells and consequently might interfere with light transmission. Alternatively, a similar oil layer might act as an additional sorption reservoir, reducing the agonists' bioavailability and hence causing a reduced response.

Figure 2.3 shows that, although the potency of the oils (based on the EC50 values) is much lower than that of B*a*P and TCDD (discussed later), the maximum luciferase induction by oils can be much higher than that of B*a*P. Supermaximal inductions were observed after the 6-h exposure period for BIL, the two bunker oils (B180 and B500), and all crude oils (AC, RC, OC, and HMC). Supermaximal responses can be explained by the presence of a chemical with higher binding affinity for the receptor or by mixture effects, as suggested by Bohonowych et al. [33]. Considering that the oils are complex mixtures of thousands of compounds, both explanations are plausible here. Compounds in the mixture might either stimulate the AhR or induce other non-receptor-mediated pathways, resulting in increased

gene expression. For instance, it has already been demonstrated that pretreatment of HepG2 101L cells with a protein kinase C activator enhanced *CYP 1A1* gene transcription induced by various AhR ligands [34]. Mixture effects are discussed later in more detail.

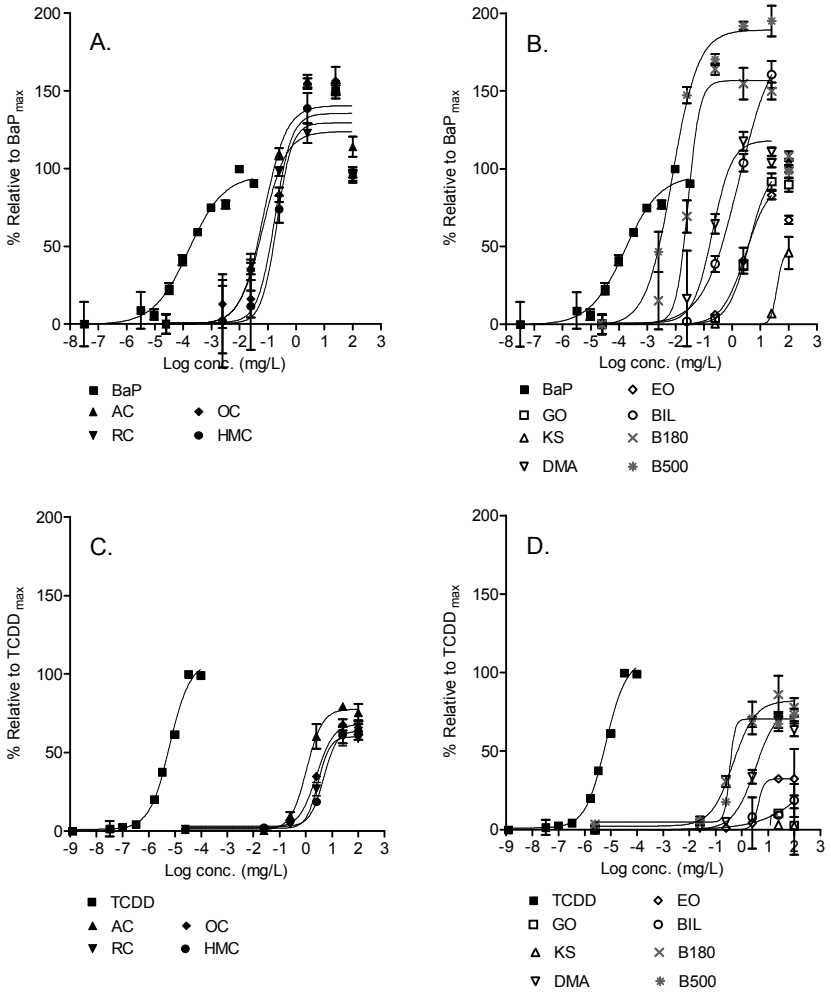

Figure 2.3: Aryl hydrocarbon receptor activation in rat hepatoma (H4IIE.luc) cells by crude oils after 6 h (**A**) and after 24 h (**C**) and by refined petroleum products after 6 h (**B**) and after 24 h (**D**). Data (± standard deviation, *n* = 3) are expressed as percentages relative to the maximum response of the standard B*a*P and TCDD for 6- and 24-h exposure, respectively. Abbreviations are explained in Table 2.1.

It is beyond the scope of the present study to speculate on the chemical nature of the compounds causing the effects observed in Figure 2.3; this topic will be investigated further in a follow-up paper. Conclusions that can be drawn from Figure 2.3, however, are that the oils probably contain different types of AhR agonists and that a single chemical is not responsible for all effects observed. After all, for some oils, responses observed at 6 h disappeared after 24 h, indicating metabolizable agonists, whereas in other cases, responses stayed high after 24 h, indicating more stable agonists. We have to note that partly disappearing responses were also observed, perhaps suggesting a mixture of both labile and stable compounds. Compounds capable of still inducing after 24 or 48 h will be persistent chemicals that are not easily metabolized. They may, therefore, also persist in the environment for prolonged periods, which makes them and their carrier oil relevant from an environmental risk assessment point of view.

Table 2.3: Potency of oils relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) following 24-h exposure in the DR-Luc assay.

Standard/oil	LEC[a](mg/L)	Relative potency[b] (LEC)	EC50[c](mg/L)	IEQ_{TCDD}[d] (µg/g)	Max response[f] (%)	Conc.max[g] (mg/L)
TCDD	3.7E-08	1	6.3E-06	1.0E+06	100 ± 1.2	4.0E-05
Refined products						
Gasoline	7.7E+00	4.9E-09	NC[g]	NC[g]	11 ± 2.7	25
Kerosene	2.5E+01	1.5E-09	NC[g]	NC[g]	3 ± 15.8	25
Distillate marine grade A	7.1E-02	5.2E-07	3.3E+00	1.9E+00	70 ± 5.1	25
Engine oil	1.4E+00	2.6E-08	NC[g]	NC[g]	32 ± 1.9	25
Bilge oil	5.5E-01	6.7E-08	NC[g]	NC[g]	19 ± 10.5	100
Bunker 180	1.8E-02	2.1E-06	4.7E-01	1.4E+01	86 ± 12.1	25
Bunker 500	1.4E-02	2.7E-06	3.5E-01	1.8E+01	73 ± 3.9	100
Crude oils						
Arabian crude	5.5E-02	6.8E-07	1.1E+00	6.0E+00	79 ± 1.0	25
Romanian crude	6.6E-02	5.6E-07	2.9E+00	2.2E+00	61 ± 6.3	25
Oseberg crude	4.2E-02	8.8E-07	2.5E+00	2.5E+00	68 ± 3.6	25
Hollmix crude	7.2E-02	5.2E-07	4.6E+00	1.4E+00	63 ± 5.0	100

[a] Lowest-effect concentration (LEC); test concentration at which first effects are observed.
[b] Ratio of the dose of TCDD and that of oil needed to achieve the LEC.
[c] Concentration at which 50% of maximum luciferase induction is reached.
[d] TCDD induction equivalents; number of micrograms of TCDD present in 1 g of oil.
[e] Percent luciferase induction (± standard deviation) calculated as the maximum response of oil relative to the maximum response for TCDD.
[f] Concentration oil at which the maximum response is reached.
[g] NC = not calculated; maximum effect not reached.

BaP- and TCDD- like potency of oils

As mentioned earlier, the potencies (as based on EC50 comparisons) of the oils tested were much lower compared to those of B*a*P and TCDD. After 6 h of exposure, the EC50s of refined oils were approximately two (B500) to four (GO) orders of magnitude higher than the EC50 of B*a*P. For the crude oils, all estimated EC50 values were similar, indicating potencies that were approximately three orders of magnitude lower than that of B*a*P. After 24 h of

exposure, EC50 values of the bunker and crude oils and DMA were approximately five to six orders of magnitude higher than that of TCDD. Note that a 6-h EC50 value for KS and a 24 h-EC50 value for GO, KS, engine oil (EO), and BIL could not be calculated due to the lack of (maximum) AhR induction at these time points. However, based on comparisons of the LECs (Tables 2.2 and 2.3), the potency of KS at 6 h was six orders of magnitude lower than that of B*a*P, while the potencies of GO, KS, EO, and BIL after 24 h were approximately eight orders of magnitude lower than that of TCDD. In general, the preceding results are consistent with those reported by Ziccardi et al. [18], who also found fuel oil No. 5 (compare with the bunker oils in the present study) to be the most potent oil in H1L1.1c2 cells after a 3-h exposure, with an EC50 of four orders of magnitude higher than the EC50 of TCDD. Regular gas, unleaded, in Ziccardi et al. [18], and GO (24 h) in the present study had similar potencies, being approximately eight orders of magnitude lower than the potency of TCDD. The lower potencies observed for oils as compared to TCDD and B*a*P can at least partly be explained because the specific petrochemical AhR agonists constitute only a fraction of the bulk oils. For instance, a considerable fraction of all oils will be occupied by alkanes [35], which are not expected to have any AhR agonistic potential and therefore dilute the potency of the agonists.

All calculated $IEQ_{B\alpha P}$ and IEQ_{TCDD} values are presented in Tables 2.2 and 2.3. The bunker oils are the most potent oils tested, having the highest number of B*a*P equivalents per mass of oil, and HMC, OC, AC, and the two bunker oils contain the highest number of TCDD equivalents per mass of oil. Similar to conventional toxic equivalency values in abiotic matrixes as recommended by the World Health Organization International Programme on Chemical Safety expert panel [36], these values do not, however, have "any toxicological implications or direct use in risk assessment, but they can be a useful tool to compare concentrations within similar abiotic matrixes and can serve a prioritization function" (p 235). For instance, the present values would strongly recommend cleanup in case of a bunker oil spill, whereas a spill of KS or GO should not take precedence. As proposed by Ziccardi et al. [18], calculated $IEQ_{B\alpha P}$ and IEQ_{TCDD} values can also be used to derive acceptable daily intake values for petroleum products or may serve as a tool for assessing risks or setting protection limits in occupational health.

DR-Luc responses caused by combined exposure to oils and BaP or TCDD

As demonstrated in Figure 2.4, the addition of a fixed concentration of B*a*P or TCDD to different concentrations of oils resulted in increased luciferase production at all concentrations and both time points. When comparing measured with expected effects at 25 mg/L after 6 h of exposure, GO, KS, DMA, EO, BIL, AC, RC, and OC had responses significantly lower than expected (1.4-2.2 times). These results suggest antagonism, given that at visual inspection no cytotoxicity was observed. This type of effect has previously been described by

Aarts et al. [21], who investigated antagonism in H4IIE.luc cells exposed to different concentrations of 2,2',5,5'-tetrachlorobiphenyl in combination with 5 pM of TCDD.

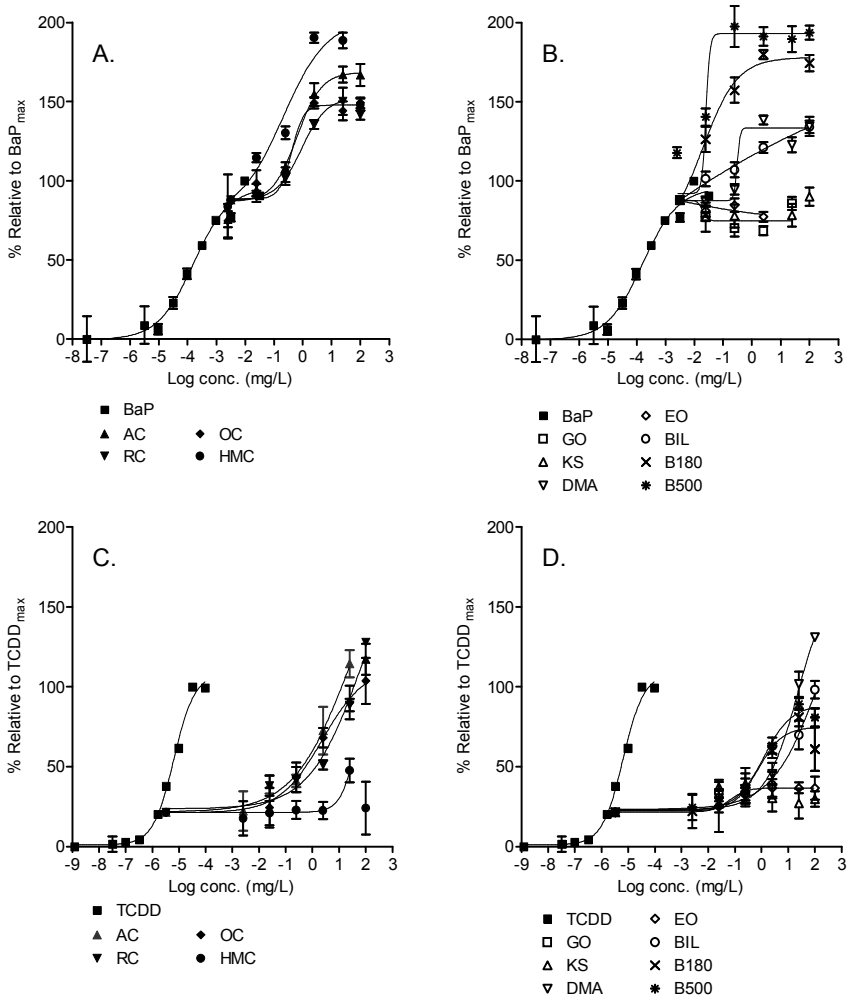

Figure 2.4: Aryl hydrocarbon receptor activation in rat hepatoma (H4IIE.luc) cells by crude oils after 6 h (**A**) and 24 h (**C**) and by refined petroleum products after 6 h (**B**) and 24 h (**D**). Cells were exposed to oils (0.025-100 mg/L) plus 3.2 x 10^{-3} mg/L of benzo[*a*]pyrene (B*a*P) for 6-h exposure or 3.2 x 10^{-6} mg/L of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 24-h exposure. Data (± standard deviations, *n* = 3) are expressed as percentages relative to the maximum response of the standard B*a*P and TCDD for 6- and 24-h exposure, respectively. Abbreviations are explained in Table 2.1.

The tetrachlorobiphenyl was shown to bind to the AhR and to subsequently prevent the binding of TCDD to the receptor [21]. Considering the complex composition of oils, multiple antagonists and compounds with other mixed interactions are expected to be present within one oil sample. Measured responses after 6 h did not differ from expected ones for HMC, B180, and B500. These results indicate an additive mode of action and therefore suggest only the presence of petroleum compounds that activate the AhR, in contrast to the chemicals causing antagonism in the previously mentioned oils. After 24 h of exposure, KS, DMA, EO, BIL, RC, OC, HMC, B180, and B500 had responses significantly lower than expected (1.2-4.3 times), again suggesting antagonism, whereas GO and AC yielded measured responses identical to expected ones, pointing to additive effects.

The results of the present study once more demonstrate the importance of investigating realistic mixtures of chemicals in addition to testing individual compounds [37,38]. During its lifetime, an organism will be exposed to mixtures of chemicals, and individual chemicals may affect one another's interaction potential with cellular receptors or specific induction pathways. Aryl hydrocarbon receptor-mediated effects upon environmental or occupational exposure to oils seem possible; although the concentrations at which effects were observed are relatively high (LECs were observed in the low microgram-per-liter range for bunker and crude oils). However, such concentrations may be attainable in organisms exposed to an oil spill or refinery effluent discharges [39]. Ultimate risks will be determined by factors like the presence of other compounds (PAHs or dioxins), the sensitivity of the species, and environmental processes controlling exposure (weathering of the oil [19] and bioavailability of the soluble fraction) as well. Risk assessment of oil is therefore a complicated task.

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Chapter 3

Specific in vitro toxicity of crude and refined petroleum products: 2. Estrogen (α and β) and androgen receptor-mediated responses in yeast assays

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Abstract

The present study is the second in a series aiming at a systematic inventory of specific toxic effects of oils. By employing a recombinant yeast stably transfected with human estrogen receptor-α (ERα) or -β (ERβ) or androgen receptor (AR) and expressing yeast enhanced green fluorescent protein, the (anti-)estrogenicity and (anti-)androgenicity of 11 crude oils and refined products was studied. None of the oils tested had significant estrogenic effects in the ERα assay or androgenic effects in the AR assay. However, all oils were capable of inducing estrogenic responses in the ERβ assay, with several responses being above even the maximal response of the standard 17β-estradiol (E2). Based on the lowest effect concentrations, the potencies of oils in all the assays were between four and seven orders of magnitude lower than those of the standards E2 or testosterone (T). The potencies of the actual individual petrochemical agonists may, however, be relatively high, considering the complex composition of oils. Additive effects, antagonistic effects, and a synergistic effect were measured in the assays upon coexposure to a fixed concentration of standard (E2 or T) and increasing concentrations of oils. To investigate whether the observed effects were receptor-mediated, coexposures to the synthetic inhibitors ICI 182,780 (ERβ assay) or flutamide (AR assay), a fixed concentration of standard, and various concentrations of oils were performed. The results suggested that the androgenic effects were receptor mediated, whereas the estrogenic effects may be only partially mediated via the receptor. The present study indicates that oils contain compounds with possible endocrine-disrupting potential, some of them acting via the hormone receptors.

Introduction

Environmental risk assessment of oils is usually performed under the assumption that the complex mixtures cause non-specific toxicity or narcosis only. However, several reports indicate that, in addition to such baseline toxicity, oils might cause a variety of specific effects. For instance, genotoxic [1], dioxin-like [2,3], (anti-)estrogenic, and (anti-)androgenic [4-6] effects have been observed. The current paper is the second in a series aiming to describe systematically the specific receptor-mediated in vitro effects of oils and the fractions thereof. After focusing on dioxin-like effects [2], the objective of the present study was to determine the estrogenicity and androgenicity of 11 different crude oils and refined petroleum products. Specific in vitro antiandrogenic and (anti-)estrogenic effects have already been described for some crude oils, a waste product, and produced water from offshore platforms [4-8], but information on refined products and the involvement of hormone receptor subtypes on the effects is lacking.

It is generally believed that chemicals might affect the endocrine system through mimicking or antagonizing the effects of endogenous hormones, affecting the levels of receptors in the corresponding tissues, and/or altering the synthesis and metabolism of

endogenous hormones [9]. A disruption of the steroid hormone homeostasis can result in several types of reproductive and developmental abnormalities in humans and in wildlife, as reviewed by McLachlan [10]. Estrogens are the main female steroid hormones, and their most important roles in vertebrates are in developmental and reproductive processes. Also, they influence many tissues such as bones, arteries, and brain. 17β-Estradiol (E2) is the major endogenous estrogen in nonpregnant, premenopausal women. In the blood, estrogens are mostly transported bound to sex hormone binding globulins. The nonbound forms of estrogens can exert their action by binding to the intracellular estrogen receptors (ER). To date, estrogen receptor-α (ERα), -β (ERβ), and –γ (ERγ) are the receptor isoforms that have been identified, with their expression differing among tissues and species [11,12]. The ERγ subtype has been identified only in fish [12]. It has recently been postulated that the extent to which these receptors are expressed in tissues ultimately can have an effect on proliferation or inhibition of cancer cells [13]. Androgens are the main male steroid hormones, which play critical roles in sexual differentiation and puberty, being important as well for the maintenance of bone, brain, muscle, skin, and hair. The action of the most potent endogenous androgen hormone, dihydrotestosterone (DHT), is mediated via the androgen receptor (AR), which is more abundant in male sex tissues than in female ones [14]. In cichlid fish, two subtypes of AR have been identified, α and β, and their expression is localized predominantly in the brain and pituitary [15]. In humans, the two forms of the AR are present in genital skin fibroblasts [16].

In the present study, the estrogenicity and androgenicity of a series of oils were determined by quantification of the enhanced green fluorescent protein activity being expressed upon activation of human ERα, ERβ, or AR by the oils in yeast recombinant estrogen and androgen assays [17,18]. Although not having metabolic capabilities like mammalian in vitro assays, yeast assays provide a fast and robust way of investigating the possible interference of chemicals with a specific endocrine receptor [19]. Other effects such as antagonism (antiestrogenicity and antiandrogenicity) or additivity were investigated with the assays by dosing the yeast with oils in combination with the receptors' natural agonists E2 or testosterone (T) at their 50% effective concentration (EC50) levels. Moreover, to determine whether the observed effects were receptor-mediated, combination exposures with the antiestrogenic compound fulvestrant (ICI 182,780; ICI) and the antiandrogenic compound flutamide (FLU) were performed.

Materials and Methods

Chemicals

All chemicals used were >98% pure, unless stated otherwise. Solvents used were ethanol (LiChrosolv grade; Merck), toluene (Spectranal grade; Riedel-de Haën), hexane and acetone (Pestiscan grade; Labscan). Testosterone, 17β-estradiol, flutamide, ICI 182,780,

ammonium sulfate, L-leucine, and histidine monohydrate were purchased from Sigma-Aldrich. Yeast nitrogen base without amino acids and ammonium sulfate, dextrose, and bacto agar were from Becton Dickinson.

Oils and dilutions

The oils investigated in the present study included seven refined products (gasoline [GO], kerosene [KS], distillate marine grade A [DMA], engine oil [EO], bilge oil [BIL], bunker oil 180 [B180], and bunker oil 500 [B500]) and four crude oils (Arabian crude [AC], Romanian crude [RC], Oseberg crude [OC], and Hollmix crude [HMC]). Their suppliers/sources, along with their applications have been given in the preceding paper [2]. All oils were dissolved 1:10 (to ~100 g/L) in toluene. Subsequently, these stocks were diluted 1:5 down to 1:200 in ethanol, resulting in oil concentrations of approximately 0.5 to 20 g/L.

Yeast

The recombinant yeast used in the present study was constructed by T. Bovee (RIKILT, Wageningen University) by stably transfecting human ERα, ERβ, AR, and yeast enhanced green fluorescent protein (yEGFP) as a reporter protein [18,20].

Yeast culturing conditions

Two days before the actual experiments, cultures were prepared by inoculating the yeast on agar supplemented with either L-leucine (for ERα and AR recombinant yeast) or histidine (for ERβ recombinant yeast). The agar plates were incubated at 30^{o}C until the first use and then stored at 4^{o}C for maximally one month. One day before exposure, overnight cultures were prepared by inoculating one colony of yeast in 15 ml of minimal medium (MM; 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, and 20 g/L dextrose) supplemented with 6 mg/ml L-leucine (MM/L) or 2 mg/ml histidine (MM/H). The culture was subsequently incubated overnight on a plate shaker at 30^{o}C and 225 rpm. During the late log phase, the culture was diluted in the medium (MM/L or MM/H) to such an extent that the optical densities at 630 nm were 0.04 for ERα and AR, and 0.1 for ERβ recombinant yeast.

Yeast exposure

Prior to exposure, 200 µl yeast suspension was plated in the 60 inner wells of a 96-well plate with V-shaped bottom (Greiner). The outer wells were filled with 200 µl sterile water. Then, 2 µl oil dilution was added directly into the pure yeast suspension in the agonism studies or into yeast suspension already containing the EC50 of either E2 or T in ethanol (i.e. 0.6 nM E2 for ERα; 0.11 nM E2 for ERβ, and 40 nM T for AR recombinant yeast) in the

combination experiments. In the mechanistic studies, 2 µl oil dilution, and either 2 µl ICI (for ERβ assay; 60.67 mg/L [0.1 mM] in ethanol) or 2 µl FLU (for AR assay; 27.26 mg/L [0.1 mM] in ethanol) solution was directly pipetted into 200 µl yeast suspension already containing the EC50 of E2 or T. In all experiments, the final ethanol concentration was below 2%. Pilot experiments had shown that this concentration was not toxic to the yeast and was not influencing the fluorescence formation as compared with the medium control. On each plate, the following controls were included: a full concentration curve of the standards, solvent controls (1 or 2% of ethanol), medium control, 20 nM T as a negative control for the ERα and ERβ assays, and 0.6 nM E2 as a negative control for the AR assay. All sample concentrations and controls were tested in triplicate in one experiment, and each experiment was replicated three times. At the end of the exposure time (6 h for ERβ assay and 24 h for ERα and AR assay), the fluorescence was measured at λ_{ex} = 485 nm and λ_{em} = 530 nm on a Polar Star Galaxy fluorescence plate reader (BMG Labtech). To determine whether the oils were cytotoxic to the yeast or were enhancing growth, the optical density was measured at 630 nm. Neither of the effects was observed. After background subtraction, the results were expressed either as percentage fluorescence formation relative to the maximum induced response by the standards (agonism and combination experiments) or as absolute fluorescence (mechanistic studies).

Data analysis

The responses induced by the oils and standards were analyzed using nonlinear regression in GraphPad Prism 4 (GraphPad Software). Data were fit according to a sigmoid dose-response curve with variable slope. Furthermore, all data were analyzed using GraphPad's two sample Student's *t* test assuming equal variances (α = 0.05). The potency of the oils was determined as previously described [3], by dividing the EC50 of the standard by the EC50 of the oil. In this case, results were expressed as E2 induction equivalents (IEQ_{E2}) or T induction equivalents (IEQ_T; Eqn. 1). The values obtained represent the number of micrograms of E2 or T equivalents present in 1 g oil:

$$IEQ_{E2} = \frac{EC50_{E2}}{EC50_{oil}} \qquad IEQ_T = \frac{EC50_T}{EC50_{oil}} \qquad (1)$$

The fluorescence production elicited by the solvent plus three times its standard deviation was considered the assay's limit of quantification (LOQ). For determining the lowest effect concentrations (LECs) of the oils, the LOQ was interpolated in the concentration-response curves of the oils.

Results and Discussion

Responses of ERα and ERβ recombinant yeast to oils

To determine ERα or ERβ activation by crude oils and refined petroleum products, the yeast was exposed for 24 and 6 h, respectively, to increasing concentrations of oils. Following a classification of estrogens as described by Andersen et al. [21] and Wang et al. [22], oils were considered fully, partially, weakly, or not estrogenic if their responses were > 75%, 25 to 75%, 10 to 25%, or <10% of the fluorescence formation by E2, respectively. As depicted in Figure 3.1A and D, no significant responses were measured in the ERα recombinant yeast for any of the oils. The highest fluorescence signal, of approximately 7% of the maximum measured for E2, was recorded for the Hollmix crude oil (HMC) at a concentration of 100 mg/L (Fig. 3.1D and Table 3.1).

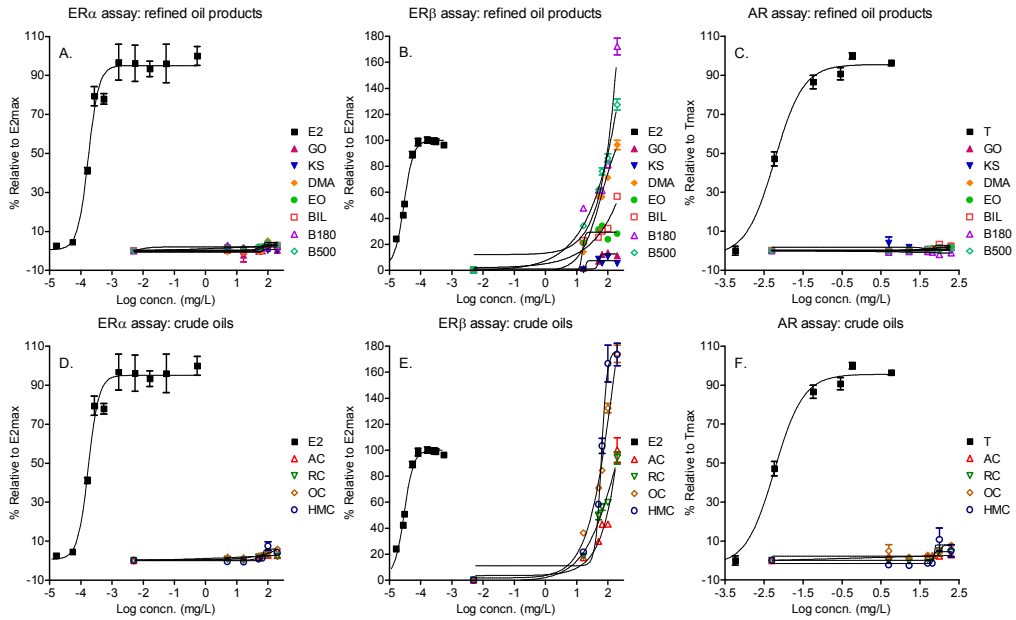

Figure 3.1: Effects of different concentrations of gasoline (GO), kerosene (KS), distillate marine grade A (DMA), engine oil (EO), bilge oil (BIL), Arabian crude light (AC), Romanian crude (RC), Oseburg crude (OC), Hollmix crude (HMC), Bunker 180 (B180), and Bunker 500 (B500) in the estrogen receptor-α (ERα; **A,D**), estrogen receptor-β (ERβ; **B,E**), and androgen receptor (AR; **C,F**) assay. Data (± standard deviation; *n* = 3) are presented as percentage fluorescence formation relative to the maximum effect induced by the standard 17β-estradiol (E2) or testosterone (T).

In contrast, in the ERβ assay, all oils were capable of activating the receptor. In this case, the responses ranged from approximately 10% of the maximum E2-induced response for KS at 100 mg/L to approximately 170% for bunker 180 (B180) and the crudes HMC and OC, all at 200 mg/L (Fig. 3.1B and E and Table 3.1). Note that, in the case of DMA, BIL, AC, RC, OC, B180, and B500, a plateau was not reached. Testing higher concentrations was not an option in these cases because of solubility constraints. The 50% effective concentrations (EC50s) for some of these oils were calculated in GraphPad Prism, assuming a theoretical maxium response. The fact that the maximum responses observed for GO, KS, EO, and BIL were <100% may be caused by the presence of partial (less effective than E2) agonists, which would induce only suboptimal conformational changes in the ERβ by binding to a secondary binding site [23]. Solubility problems are not plausible, insofar as these cases concern the light, refined oils only.

Table 3.1: Estrogenic potency of oils relative to 17-β estradiol, as calculated based on their responses in the estrogen receptor-α (ERα) and -β (ERβ) assays

	ERα						ERβ					
Standard/oil	LEC[a] (mg/L)	Relative potency[b] (LEC)	EC50[c] (mg/L)	IEQ_{E2}[d] (µg/g)	Max response[e] (%)	Conc. max[f] (mg/L)	LEC[a] (mg/L)	Relative potency[b] (LEC)	EC50[c] (mg/L)	IEQ_{E2}[d] (µg/g)	Max response[e] (%)	Conc. max[f] (mg/L)
17β-Estradiol	6.0E-06	1	1.7E-04	1.0E+06	100 ± 4.9	0.545	9.7E-06	1	2.9E-05	1.0E+06	100 ± 2.4	0.0002
Refined products												
Gasoline	1.1E+02	5.7E-08	NC	NC	1.29 ± 0.3	100	4.9E+00	2.0E-06	NC	NC	13.5 ± 0.6	100
Kerosene	1.3E+00	4.6E-06	NC	NC	1.47 ± 0.6	5	1.8E+01	5.5E-07	NC	NC	10.2 ± 0.4	100
Distillate marine grade A	7.0E+01	8.6E-08	NC	NC	5.27 ± 0.6	100	4.7E+00	2.1E-06	83.82	3.5E-01	96.4 ± 3.5	200
Engine oil	5.0E+00	1.2E-06	NC	NC	3.12 ± 1.0	100	7.2E+00	1.3E-06	NC	NC	34.2 ± 1.1	66
Bilge oil	5.2E+01	1.2E-07	NC	NC	3.29 ± 0.5	100	4.8E+00	2.0E-06	NC	NC	56.7 ± 1.1	200
Bunker 180	8.2E-01	7.3E-06	NC	NC	2.10 ± 1.0	100	4.5E-02	2.2E-04	113.9	2.5E-01	172.1 ± 6.4	200
Bunker 500	3.6E+01	1.7E-07	NC	NC	4.33 ± 0.4	100	5.0E+00	1.9E-06	138.9	2.1E-01	127.4 ± 4.4	200
Crude oils												
Arabian crude	1.8E+00	3.4E-06	NC	NC	2.78 ± 0.5	100	4.9E+00	2.0E-06	95.58	3.0E-01	100.1 ± 9.5	200
Romanian crude	4.0E+01	1.5E-07	NC	NC	2.94 ± 0.5	100	1.5E+01	6.5E-07	57.61	5.0E-01	94.1 ± 4.7	200
Oseberg crude	1.7E+01	3.6E-07	NC	NC	5.86 ± 1.0	200	4.0E+00	2.4E-06	142.9	2.0E-01	174.1 ± 6.7	200
Hollmix crude	6.8E+01	8.9E-08	NC	NC	7.47 ± 2.2	100	2.4E+01	4.1E-07	61.64	4.7E-01	173.7 ± 8.5	200

[a] Lowest effect concentration; test concentration at which first effects are observed.
[b] Ratio of the dose of estradiol and that of oil needed to achieve the LEC.
[c] Concentration at which 50% of maximum fluorescence induction is reached.
[d] Estradiol induction equivalents; number of micrograms of E2 present in one gram of oil.
[e] Percentage fluorescence induction (± standard deviation) calculated as the maximum response of oil relative to the maximum response for estradiol.
[f] Concentration oil at which the maximum response is reached.
NC = not calculated; maximum effect not reached.

To the best of our knowledge, this is the first report on specific ER binding and activation by pure crude oils and refined petroleum products in recombinant yeast assays. Reports do exist, however, on the estrogenic activity of offshore water effluents from North Sea oil production platforms in a recombinant yeast-based estrogen assay [8,24,25]. The

authors attributed the observed agonist activity to the presence of petrogenic alkyl phenols. Given that yeast is lacking the normal metabolic pathways found in mammalian cells [18], the observed effects must be caused by parent petroleum compounds and not by, e.g., hydroxylated polycyclic aromatic hydrocarbons, the biotransformation products of polycyclic aromatic hydrocarbons (PAHs) that are well known for their estrogenic activity [26].

The pure oils tested in the present study caused some interesting responses: both specific induction of a single receptor isoform and responses exceeding the E2 maximum were observed. Nevertheless, similar effects have been described for individual chemicals. First, different activation of the two ER isoforms has been reported for phytoestrogens and other individual chemicals [27,28]. For example, Fertuck and coworkers [27] showed a higher ERβ-dependent gene expression in mammalian cells treated with several PAHs. However, considering the large difference in the ligand-binding domain between the two ERs, it is not surprising that chemicals bind to the receptors with a different affinity [29]. Second, supermaximal responses (i.e., induction of fluorescence formation to higher levels than E2 does), as observed in the present study for HMC, OC, and the bunker oils, have previously been reported for certain pesticides, hydroxylated PAHs, and bisphenol A in mammalian reporter gene assays [26,30,31]. The authors hypothesized that a stimulation of the receptor concentration, cofactor renewal, and/or a modulation of several kinases involved in estrogen-responsive elements activation might be the cause for the observations.

The ultimate biological effects of potential xenoestrogens will depend, among other things, on the tissue distribution of the two ERs and the extent to which they are expressed in a certain tissue [13]. This might explain the variability in estrogenic effects described for oils. For example, female mussels exposed to environmentally relevant concentrations of North Sea oil had enlarged ovarian follicles [32]; female fish exposed to waste products of Athabasca oil sands mining had reduced plasma E2 levels compared with control female fish [33]; and male fish exposed to naphthenic acids (constituents of the mining waste) had increased plasma E2 levels [33]. All in all, the above-mentioned studies as well as the present study clearly suggest that oils have endocrine-disrupting potential. Given the possible molecular and biological differences among species, this potential would, however, require confirmation in a mammalian system before one would be able to assess final risks to organisms. Also, the lipophilicity and resistance to biotransformation will finally determine whether these estrogenic compounds bioaccumulate in vivo to concentrations high enough to produce an estrogenic effect.

Responses of AR recombinant yeast to oils

The androgenicity of oils was investigated by exposing the AR recombinant yeast for 24 h to different concentrations of the crude oils and refined petroleum products. No cytotoxicity was detected at any of the concentrations tested (data not shown). Furthermore, from all the oils tested, the highest response of approximately 11% relative to the T

maximum was measured for HMC at a concentration of 100 mg/L (Fig. 3.1C and F and Table 3.2). All other oils induced fluorescence formation below 10% and therefore should be considered not androgenic. Also, in other studies, no androgenicity was observed for oils: a heavy oil type C and water discharges from offshore production platforms did not induce effects in prostate cancer (LNCaP) cells or recombinant yeast [5,24]. This suggests a lack of androgenic activity for both crude oils and refined petroleum products, indicating the absence of petrochemicals capable of activating the AR. To date, only pharmaceuticals, diesel fuel exhausts, pulp mill effluents, and antimicrobial additives have been identified as xenoandrogens [34].

Table 3.2: Androgenic potency of oils relative to testosterone, as calculated based on their responses in the androgen receptor assay

Standard/oil	LEC[a] (mg/L)	Relative potency[b](LEC)	EC50[c] (mg/L)	IEQ_T[d] (µg/g)	Max response[e](%)	Conc.max[f] (mg/L)
Testosterone	4.8E-05	1	5.8E-03	1.0E+06	100 ± 1.2	
Refined products						
Gasoline	NC	NC	NC	NC	1.64 ± 1.3	100
Kerosene	NC	NC	NC	NC	3.82 ± 3.2	5
Distillate marine grade A	NC	NC	NC	NC	0.77 ± 0.6	200
Engine oil	6.5E+01	9.3E-08	NC	NC	2.30 ± 0.8	200
Bilge oil	7.0E+01	8.6E-08	NC	NC	3.26 ± 0.6	100
Bunker 180	6.5E+01	9.3E-08	NC	NC	1.11 ± 0.3	200
Bunker 500	6.7E+01	8.9E-08	NC	NC	1.69 ± 1.5	200
Crude oils			NC	NC		
Arabian crude	2.5E-01	2.4E-05	NC	NC	2.83 ± 0.6	200
Romanian crude	6.8E+01	8.9E-08	NC	NC	4.90 ± 0.6	100
Oseberg crude	2.2E+01	2.7E-07	NC	NC	7.83 ± 0.9	200
Hollmix crude	7.1E+01	8.5E-08	NC	NC	10.7 ± 5.9	100

[a] Lowest effect concentration; test concentration at which first effects are observed.
[b] Ratio of the dose of testosterone and that of oil needed to achieve the LEC.
[c] Concentration at which 50% of maximum fluorescence induction is reached.
[d] Testosterone induction equivalents; number of micrograms of T present in one gram of oil.
[e] Percentage fluorescence induction (±standard deviation) calculated as the maximum response of oil relative to the maximum response for testosterone.
[f] Concentration oil at which the maximum response is reached.
NC = not calculated; maximum effect not reached.

Responses of ERα and ERβ recombinant yeast to oils plus E2

Other biological effects of oils (e.g., antagonism) were investigated by dosing the yeast with different concentrations of oils plus a fixed concentration of E2. These fixed E2 concentrations, the EC50 of E2 in the assays, were 0.6 nM (1.63×10^{-4} mg/L) for ERα and 0.11 nM (3×10^{-5} mg/L) for ERβ recombinant yeast. To test whether the resulting combined responses should be considered additive, antagonistic, or synergistic, comparisons between measured and expected responses were performed using the response addition approach [2]. A concentration addition approach is less appropriate in this case, because it is unclear whether all chemicals from the complex mixtures that are contributing to the overall

responses are acting according to the same mode of action (see, e.g., earlier discussion on partial and supermaximal responses). For ERα recombinant yeast, the responses obtained for most of the oils were not significantly different from the EC50 of E2, indicating the absence of combination effects for the oils. However, in case of 200 mg/L BIL and RC, responses of approximately 26% and 20% of the E2 EC50 response were measured, respectively. These responses were significantly (p < 0.01) lower than the E2 EC50, suggesting ER antagonism (Fig. 3.2A and D).

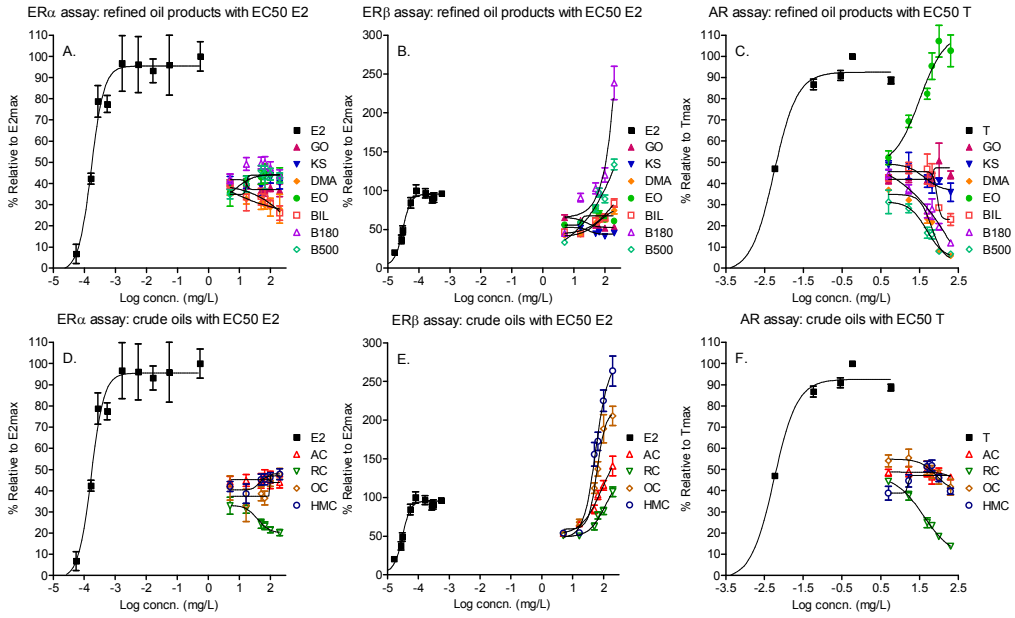

Figure 3.2: Responses in the estrogen receptor-α (ERα; **A,D**), estrogen receptor-β (ERβ; **B,E**), and androgen receptor (AR; **C,F**) assays after coexposure of yeast to oils and the EC50 of 17β-estradiol (E2; ER assays) or testosterone (T; AR assay). Data (± standard deviation; n = 3) are presented as percentage fluorescence formation relative to the maximum effect induced by the standard E2 or T.

Arcaro and co-workers [4] demonstrated antiestrogenicity of a clarified slurry oil (a constituent of bunker oils) and two crude oils in the human breast cancer cell (MCF-7) assay. The two possible mechanisms to explain these effects are an increased catabolism of E2 and competition between petrochemicals and E2 for binding to the ER. Because the yeast used in the present study is devoid of metabolic enzymes and other receptors, the presently observed effects can be explained only through interaction with the receptor. Possible competitors may be three- to seven-ring PAHs, as suggested by Arcaro et al. [4].

The results of the exposure of the ERβ recombinant yeast to increasing concentrations of oils and a fixed concentration of E2 suggested additive effects (Fig. 3.2B and E). Responses exceeding the maximum response of E2 were measured in case of AC, OC, HMC, B180, and B500 and were 141, 206, 264, 239, and 134%, respectively (Fig 3.2B and E). Possible mechanisms involved will be discussed in more detail below.

Responses of AR recombinant yeast to oils in combination with T

Exposure of the AR recombinant yeast to increasing concentrations of oils plus a fixed concentration of T (40 nM; 1.1×10^{-2} mg/L) had no effect in the case of GO, KS, AC, OC, and HMC, and antagonistic effects in the case of DMA, BIL, RC, B180, and B500 (Fig. 3.2C and F). For the oils antagonizing the effect of T, the response was reduced to 6% and 23% of the T-induced response by DMA and BIL, respectively. Using another recombinant yeast assay, Tollefsen et al. [8] demonstrated the presence of AR antagonists in produced water effluents resulting from oil offshore platforms. Although the chemical nature of the AR antagonist was not identified, a petrogenic source cannot be excluded. Antiandrogenicity of heavy oils has been shown in mammalian cells, as well as in mouse mammary carcinoma (SC115) and human prostate carcinoma (LNCaP) cells, and it has been associated with the presence of certain petrogenic PAHs [5]. Environmental (anti-)androgens can have adverse effects on the reproduction of males and females, and exposure to oils might therefore potentially result in demasculinization of male offspring or abnormalities in sexual development. This type of adverse effects has previously been observed for 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), 4,4′-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), and the fungicide vinclozolin and its metabolites [35]. Still, one should be careful classifying oil as an antiandrogen, insofar as Kizu et al. [36] have noted that the antiandrogenic effects seen in LNCaP cells can be due to cross-talk with the aryl hydrocarbon receptor. In these cells, an activator protein was expressed, which finally inhibited the binding of AR to androgen-responsive elements.

Remarkably, responses of approximately 95, 107, and 102% of the T-induced response were measured in the present study when the yeast was coexposed to 5.83×10^{-3} mg/L T and 66, 100, or 200 mg/L of EO, respectively (Fig 3.2C). These responses were significantly higher than the ones expected based on the sum of the measured responses of EO at 66, 100, or 200 mg/L alone and the EC50 of T. After all, EO alone did not induce significant responses (see Fig. 3.1C), and the expected response upon coexposure to the EC50 of T thus measures approximately 50%. The observed responses of 95 to 107% therefore indicate a synergistic effect. Synergism has not previously been observed for oils. Kunz and Fent [23] have, however, described synergism in a recombinant ERα yeast assay occurring at low-dose mixtures of ultraviolet filters. The authors hypothesized that compounds in the mixture may activate and coactivate the receptor. We do not have a conclusive explanation for the

abnormal behavior of the particular EO oil in the present study, but specific additives in the commercial product might be involved.

E2 and T-like potency of oils

Because of the absence of estrogenic and androgenic responses for the oils in the ERα and AR assay, EC50s could not be calculated for the oils (Tables 3.1 and 3.2). The same applied to GO, KS, EO, and BIL in the ERβ assay (Table 3.1). To remain able to quantify the potency of the oils, the lowest effect concentration (LEC) was calculated where possible. In the ERα assay, the resulting LEC values were five to seven orders of magnitude higher than the LEC value of E2, indicating potencies five to seven orders of magnitude lower than that of E2 (Table 3.1). In the ERβ assay, the potencies of the oils were four to six orders of magnitude lower than the potency of E2. In case of the AR assay, AC had a potency of four orders of magnitude lower than that of T, whereas the other oils had potencies that were approximately six orders of magnitude lower (Table 3.2). As mentioned previously [2], it should be stressed that the oils are extremely complex mixtures of thousands of chemicals. A potency being four to seven orders of magnitude lower than that of the standard may therefore still imply the presence of highly potent petrochemical agonists.

The annual, worldwide natural seepage of crude oil into the marine environment is estimated to be 0.2 to 2 million metric tons, with a best estimate of 0.6 million metric tons [37]. Assuming a concentration of E2 equivalents of 3.6×10^{-1} µg/g crude oil (average potency of the crude oils; Table 3.2), the annual input resulting solely from natural sources would be 220 kg of E2 equivalents. However, natural seepage is not the only source of oil in the (marine) environment. Other sources such as sewage effluents, accidental spills, and discharges from offshore platforms will add up to the overall load of petrochemical xenoestrogens and antiandrogens into the environment. For instance, Tollefsen and co-workers [8] have estimated an annual 560,000 kg of flutamide (antiandrogen) equivalents being discharged into the North Sea, solely from offshore platforms.

Receptor-mediated effects

To investigate whether the responses observed upon exposure to oils in combination with the assays' positive standards were mediated via the receptors, EO (AR assay) and DMA, BIL, RC, B180, and B500 (ERβ assay) were studied in more detail. Well-known inhibitors of ER and AR, i.e., ICI and FLU, respectively, were added at their maximum inhibiting concentrations (0.1 mM) to the media containing 200 mg/L oil or to media containing oil plus the E2 or T EC50. Pilot experiments had indicated that 0.1 mM ICI and FLU were not toxic when combined with E2 or T (data not shown). In both cases, the estrogenic or androgenic responses were significantly reduced to almost the background level (Fig. 3.3). Furthermore,

the addition of 0.1 mM ICI to the medium containing 200 mg/L B180 significantly reduced the response to the level of oil alone. When the same concentration of ICI was added to the medium containing both 200 mg/L B180 and E2, the estrogenic response was significantly reduced compared with the control (only oil and E2). However, in neither of the last two treatments was ICI able to counteract completely the effects of the (xeno-)estrogens, even though, as stated above, it was able to inhibit completely the effect of E2. This type of partial inhibition was also observed for the other oils tested (DMA, BIL, RC, and B500; data not shown). These observations might be explained in three ways. First, the effects seen for B180 might not be fully ER mediated; second, the concentrations of petrogenic estrogens might have been too high so that ICI could only partially block their effect; or, third, specific petrogenic compounds might have prevented the down-regulation of ER protein by ICI [38].

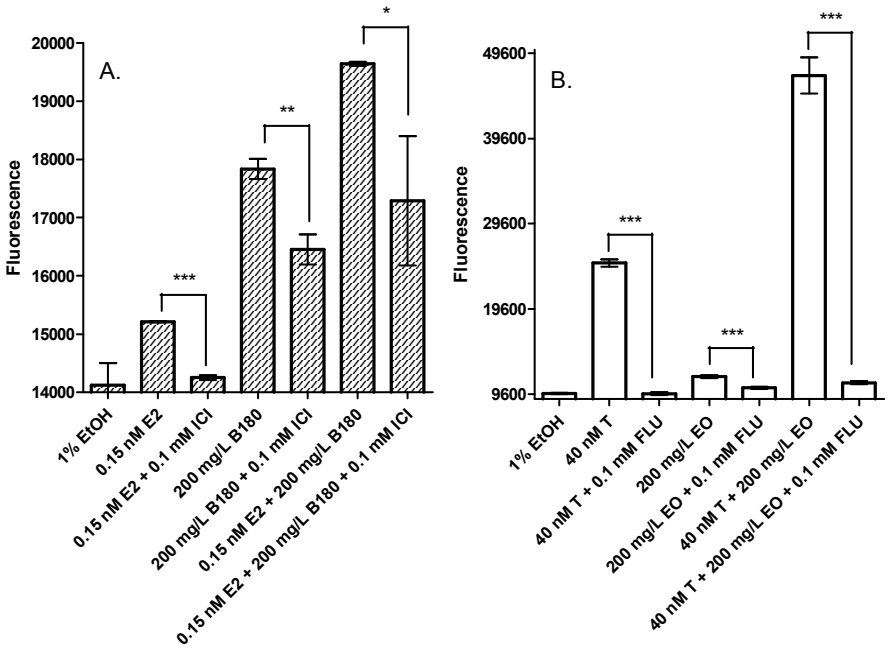

Figure 3.3: Responses upon dosing different combinations of the 50% effective concentration (EC50) of 17β-estradiol (E2), the estrogen receptor (ER) antagonist ICI 182,780 (ICI), and bunker 180 (B180), in the ERβ assay (**A**) and different combinations of the EC50 of testosterone (T), the androgen receptor (AR) inhibitor flutamide (FLU), and engine oil (EO), in the AR assay (**B**). Significantly different from the control at *$p<0.05$, **$p<0.005$, and ***$p<0.0005$.

In Figure 3.3B, the effects of the AR inhibitor FLU on the androgenic response by 40 nM T, 200 mg/L EO, and a combination of these two in the AR assay are presented. As opposed to ICI in the above-described experiment, FLU was able to inhibit the androgenic responses completely in all cases, suggesting that the effects of EO are fully receptor mediated in this assay.

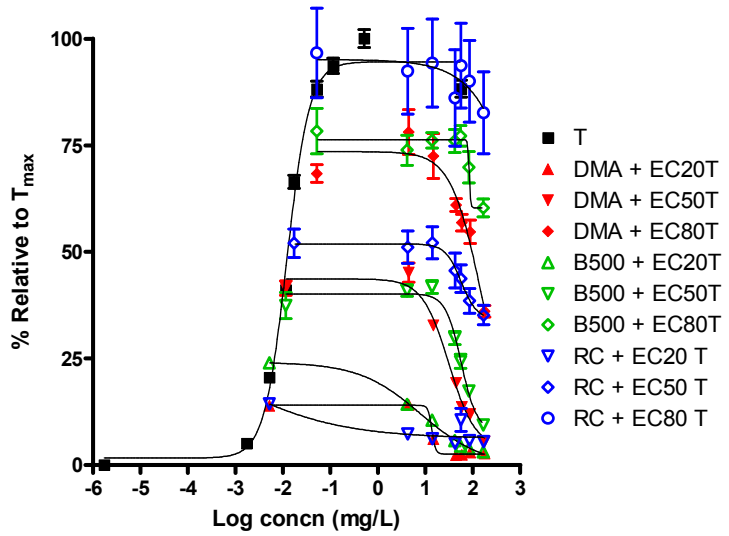

Figure 3.4: Responses in the androgen receptor (AR) assay upon coexposing yeast to different concentrations of distillate marine grade A (DMA), bunker 180 (B180), or Romanian crude (RC) plus either the 20% effective concentration (EC20) of testosterone (T) (18 nM), the EC50 of T (40 nM), or the EC80 of T (180 nM). Data (±standard deviation; *n* = 3) are presented as percentage fluorescence formation relative to the maximum effect induced by the standard T.

As a last step, the oils that produced clear antiandrogenic responses in the AR recombinant yeast when coexposed to T (i.e., DMA, RC, and B500) were dosed to the yeast at increasing concentrations in the presence of T at its EC20, EC50, or EC80 levels (i.e., 18 nM, 40 nM, and 180 nM; Fig. 3.4). A combination of different concentrations of DMA, RC, or B500 with the EC20 of T lead to a significant decrease in the androgenic response, with the first effects seen at concentrations of about 14 mg/L for DMA and RC, and 42 mg/L for B500 (Fig 3.4). When T was added at its EC50, first significant decreases in the responses were observed at approximately 14 mg/L DMA, 43 mg/L RC, and 56 mg/L B500, with the responses being reduced to 6, 35, and 9%, respectively, in the presence of the maximum concentration of the oils. By increasing the concentration of T to its EC80 level, the effects of RC and B500 on the yeast were significantly counteracted, with the first significant decrease seen at about

170 mg/L. However, in the case of DMA, the first significant decrease in the response was still noted at approximately 45 mg/L, with a maximum decrease up to 36% at the highest concentration of DMA tested.

These results suggest that, for RC and B500, the effects are strictly receptor mediated, and they can be reversed by increasing the concentration of the natural AR ligand. This hypothesis, however, does not apply to DMA. For this oil, the data obtained suggest that other pathways are involved in the antagonistic effects. Given that the recombinant yeast used is devoid of other receptors present normally in mammalian cells [18], interactions with other receptor-mediated pathways can be excluded. Possibly, DMA contains compounds that can bind to the receptor but are not capable of activating it or compounds that inhibit protein kinases present in yeast [39].

In a receptor-mediated pathway, the binding of a compound to the receptor is followed by the activation of the corresponding receptor. Subsequently, a cascade of molecular events will take place in the target cells, finally leading to altered gene expression. Studies suggest that man-made and natural compounds can disrupt the estrogen and androgen signaling pathways in humans and wildlife, starting from early embryonic life stages to adulthood [34,40], with consequences for the reproductive success. Although most of the oils tested in the present study appeared to be biologically active in the recombinant yeast assays (i.e., all except gasoline and kerosene), additional experiments would be needed to classify the oils officially as endocrine disruptors. After all, the recombinant yeast assays applied here lack the complex molecular pathways present in vertebrate cells. This topic will be dealt with in a followup study.

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Chapter 4

Specific in vitro toxicity of crude and refined petroleum products: 3. Estrogenic responses in mammalian assays

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Abstract

Current petroleum risk assessment considers only narcosis as the mode of action, but several studies have demonstrated that oils contain compounds with dioxin-like, estrogenic or antiestrogenic, and androgenic or antiandrogenic activities. The present study is the third in a series investigating the specific toxic effects of 11 crude oils and refined products. By employing recombinant mammalian cells stably transfected with the human estrogen receptor alpha (ERα) or beta (ERβ), and expressing the luciferase protein (ERα-U2OS-Luc and ERβ-U2OS-Luc assay), the estrogenicity or antiestrogenicity of oils was studied. All oils, except for two refined and one crude oil, induced estrogenic responses. The calculated estrogenic potencies of the oils were six to nine orders of magnitude lower than the potency of 17β-estradiol (E2). Upon coexposure to a fixed concentration of E2 and increasing concentrations of oils, additive, antagonistic, and synergistic effects were revealed. One nautical fuel oil was tested in the human breast carcinoma cell line (MCF-7), in which it induced cell proliferation up to 70% relative to the maximum induction by E2. At its minimum effect concentration of 25 mg/L, the oil was also capable of inducing mRNA expression of the estrogen-dependent protein pS2 by a factor of two. The present results indicate that oils naturally contain potentially endocrine-disrupting compounds that are able to influence the estrogenicity of other compounds and may cause biological responses beyond receptor binding.

Introduction

Oil pollution has been shown to affect both human and environmental health [1,2]. For example, humans living in the vicinity of oil exploitation fields have increased rates of different types of cancer [1], and petroleum hydrocarbons have been shown to cause teratogenic effects in birds and to affect their egg production and hatchability [3]. Apart from nonspecific effects, such as smothering (direct physical effect) and narcosis (disturbance of biomembrane structure and functioning) [4], oils may therefore cause specific effects as well. To study the cause of such specific effects, several in vitro assays have been employed, which has lead to the documentation of, among others, mutagenic, antiandrogenic, estrogenic, antiestrogenic, and dioxin-like effects [5-13]. The investigation of various specific effects of a broad range of oils by using in vitro assays is important for understanding the mechanisms leading to possible physiological and biological changes in organisms. This might contribute to an improvement of the risk assessment of oils.

The present study is the third in a series of papers describing several specific effects of environmentally relevant oils and the fractions thereof. In the previous paper, estrogenic and androgenic effects of oils were investigated in yeast bioassays [10]. Although these simple and fast assays suggested endocrine-disruptive properties of the oils, confirmation of the results in mammalian cells would be needed to obtain an indication of effects that might occur in mammals. Therefore, the objective of the present study was to determine the

estrogenic potential of a series of 11 crude oils and refined petroleum products in human cell-based reporter assays, as well as their effects on cell proliferation and gene expression. Receptor-mediated effects of the oils were determined by quantification of the luciferase activity expressed upon activation of human estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) in the ER-mediated luciferase reporter gene assays (ER-Luc assays) [14]. The two separate assays were used because, given the different tissue distribution of the two estrogen receptors as well as the difference in their ligand binding domains, it is important to employ assays that will strictly yield responses induced via the distinct receptors [15]. In addition to receptor agonism, possible antagonistic, additive, or synergistic effects were investigated by exposing the cells to oils in combination with the 50% effect concentration (EC50) of the assays' positive control, 17-β estradiol (E2). Cell proliferation and gene (pS2) expression as examples of more complex biological effects were investigated in the estrogen-responsive human breast carcinoma cell line MCF-7. The pS2 gene is an ER-regulated protein in MCF-7 cells [16,17]. The overall merit of the data obtained in the present study will be an improved insight into the potential of petroleum hydrocarbons to bind to specific mammalian ERs and to elicit subsequent biochemical effects. In a followup paper, the chemical nature of the causative compounds will be discussed.

Materials and Methods

Chemicals

Solvents used to dissolve the oils and clean the glassware were ethanol (LiChrosolv grade; Merck), toluene (Spectranal grade; Riedel-de Haën), and hexane and acetone (Pestiscan grade; Labscan). Chemicals used during the in vitro work were 17-β estradiol (E2), insulin, and tricine (Sigma-Aldrich); fetal calf serum (FCS; Australian origin); geneticin (Invitrogen Life Technologies); hyclone-dextran-charcoal-treated FCS (DCC-FSS; Perbio Science NV) [18]; phosphate-buffered saline (PBS; without Ca^{2+} and Mg^{2+}), nonessential amino acids (x100), 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 without phenol red, trypsin, penicillin, streptomycin, sodium pyruvate, and glutamine (Gibco); sodium hydrogen carbonate ($NaHCO_3$), sodium hydroxide (NaOH), ethylenedinitrotetra-acetic acid ($EDTA{\cdot}2H_2O$), magnesium sulfate ($MgSO_4{\cdot}7H_2O$), 1,4-dithiothreitol (DTT; Merck); d-luciferin and hygromycine (Duchefa); tris(hydroxymethyl)aminomethane (Tris; Invitrogen), *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*',*N*'-tetraacetic acid monohydrate (CDTA; Fluka); and adenosine triphosphate (Roche).

Oils and dilutions

All 11 oils used in the present study and their sources and uses have been described in part 1 of our series of papers [13]. First, the oils were dissolved 1:10 (~100 g/L) in toluene, and subsequently these stocks were diluted 1:5 and 1:20 in ethanol, resulting in oil

concentrations of approximately 20 and 5 g/L. Additional dilutions were prepared by either diluting the 100 g/L toluene stock 1:10 and 1:15 in ethanol, resulting in concentrations of 10 and 67 g/L, or diluting the 5g/L solution 1:3 in ethanol, resulting in a concentration of 1.6 g/L. All remaining test dilutions were then further diluted in ethanol as described previously [13]. The common method of in vitro dosing by means of a carrier solvent [8,9,11] was used instead of applying water-accommodated fractions, primarily not to exclude specific oil constituents a priori, that is, chemicals not being soluble or present in sufficiently high concentrations in water (accommodated fractions) but that might be taken up by organisms via oil-contaminated food, soil, or sediment.

Cell lines and cell culture conditions

Stably transfected human osteosarcoma cell lines (ERα-U2OS-Luc and ERβ-U2OS-Luc) [14] were cultured in DMEM/F12, supplemented with 1,260 mg/L $NaHCO_3$, 7.5% FCS (Australian origin), and 0.5% nonessential amino acids. As selection markers, geneticin (200 µg/ml) and hygromycin (50 µg/ml) were added to the ERα-U2OS-Luc culture medium, whereas only geneticin (200 µg/ml) was added to the ERβ-U2OS-Luc culture medium. Both cell lines were cultured at 37°C and with 7.5% CO_2 in a humidified atmosphere.

The human breast carcinoma MCF-7 cell line was obtained from the American Type Culture Collection and was cultured in DMEM without phenol red, supplemented with 5% FCS, 100 mM sodium pyruvate, 1% penicillin-streptomycin, 200 mM glutamine, and 10^{-3} M insulin at 37°C and 5% CO_2 in a humidified atmosphere.

ER-U2OS-Luc assays procedure

The reporter gene assays were performed as described previously by Sotoca Covaleda et al. [18] and ter Veld et al. [19]. The 80 to 90% confluent cells were washed with PBS, trypsinized, and then diluted to 10×10^4 cells/ml (ERα-U2OS-Luc) or 7.5×10^4 cells/ml (ERβ-U2OS-Luc) in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red but buffered with 1,260 mg/L $NaHCO_3$, 0.5% nonessential amino acids, and 5% DCC-FCS (exposure medium). After seeding, the cells were incubated for 2 d at 37°C and with 7.5% CO_2 in a humidified atmosphere until exposure to allow depletion of the E2 remaining in the culture medium. Next, the old medium was replaced with 100 µl/well of new exposure medium after 1 d. On the second day, the medium was replaced with 200 µl fresh exposure medium, which, for combination studies, also contained 3 pM E2 (ERα-U2OS-Luc) or 100 pM E2 (ERβ-U2OS-Luc). The cells were exposed to oils in triplicate by directly pipetting 1 µl of oil dilution into 200 µl medium (final solvent concentration 0.5% [v/v]). A solvent control (0.5% ethanol), an exposure medium control, and a full E2 dose-response curve were included for each plate. All experiments were repeated three times, i.e., on different days of three consecutive weeks, using different cell passages. After 24 h of exposure, the medium was removed, and the cells were washed with 100 µl of 0.5× PBS per well and then lysed with 30

µl of hypotonic low-salt buffer (10 mM Tris, 2 mM dithiothreitol, and 2 mM CDTA, pH 7.8). The plates were subsequently placed on ice for approximately 10 min to allow swelling of the cells and were then frozen at -80^{o}C for at least 30 min, until use. Just before measurement, the plates were thawed at room temperature for approximately 60 min and then shaken for another 2 min. The luciferase production was then measured in a luminometer (Labsystems, Luminoskan RS) equipped with two injection pumps. The background light emission was measured for the first 2 s. Then, 100 µl flashmix (20 mM tricine, 1.07 mM $(MgCO_3)4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM $EDTA{\cdot}2H_2O$, 2 mM DTT, 0.47 mM d-luciferin, and 5 mM ATP, pH 7.8) was added, and the light emission was measured for an additional 2 s, after which it was extinguished with 50 µl of 0.2 M NaOH [19]. After background subtraction, the responses of E2 and the oils were expressed as percentage estrogenic response relative to maximum response of E2 (E2max).

MCF-7 proliferation assay

Five days before the actual exposure, the MCF-7 cells were passaged into assay medium (phenol red-free DMEM supplemented with 1% penicillin and streptomycin, 5% serum hyclone, 200 mM glutamine, 100 mM sodium pyruvate, and 10^{-3} M insulin), and left to grow at 37^{o}C with 5% CO_2. After 3 d, the cells were trypsinized, diluted, and seeded at 8,000 cells/well using assay medium in 96-wells plates (Greiner). After an additional 2 d of incubation, the old assay medium was removed and replaced with new assay medium containing the positive control E2 (0.1 pM to 3 nM), a 0.5% ethanol solvent control, or oil dilutions at concentrations of 2.5 to 100 mg/L. Each concentration was tested in triplicate on the same plate, and the experiments were replicated four times. Although preferably a maximum solvent concentration of 0.1% should be used in mammalian cell-based assays, we used a maximum of 0.5% ethanol, because of problems with concentrating the oil stock solutions. However, pilot experiments had indicated that there was no significant difference in cell proliferation among any of the ethanol concentrations (0.1-0.5%) and the one of the medium-only control. In combination with the positive control, E2, however, the EC50 obtained with 0.1% solvent was three times lower than the one obtained with 0.5% solvent. The maximum percentage cell proliferation was achieved in both cases at 0.3 nM E2. Given these results, for the present experiments, a maximum of 0.5% ethanol was used. Pilot experiments further had indicated that the maximum proliferation was achieved after 5 d. Therefore, after 5 d of exposure, a 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as an indicator of the number and vitality of cells. The principle of this assay is based on the reduction of the yellow MTT reagent to blue formazan by the mitochondria of viable cells [20]. Fifty microliters of MTT (5 mg/L) was added to 200 µl medium from each well, resulting in a final MTT concentration of 1 mg/ml. Subsequently, the cells were incubated for an additional 40 min at 37^{o}C and 5% CO_2. Upon finishing the incubation, the medium was removed, and the formazan crystals were dissolved in 150 µl isopropanol. Formazan formation was measured at 595 nm in a spectrophotometer (BMG

POLARstar). Finally, the estrogen-induced cell proliferation was expressed relative to the proliferation induced by the solvent control (set at 100%).

pS2 gene expression assay

To determine pS2 gene expression, MCF-7 cells were plated using assay medium in a 12-well plate at a density of 2×10^5 cells/ml. After 2 d, during which the cells were allowed to attach to the bottom of the wells, the medium was removed and refreshed with 1 ml of new assay medium containing 10 nM E2, 0.1% ethanol control, or 25 mg/L oil dilution. The cells were exposed for 5 d, after which RNA was isolated from the cells according to the enclosed instructions of the applied RNA Instapure System kit (Eurogentec). The RNA concentration was assessed by measuring the ratio of the absorbance at 260 and 280 nm, where 260 nm was used to calculate the RNA concentration [21]. The RNA samples were frozen at -80^0C until further use. Next, cDNA was prepared by diluting the RNA to 66.7 µg/ml and using the ISCRIPT™ synthesis kit (Bio-Rad). For real-time polymerase chain reaction (PCR) measurements, the cDNA was diluted five times in RNAse-free water, and subsequently pS2 primer (Invitrogen) and β-actin (housekeeping gene) mix were prepared. The β-actin gene expression did not change upon treatment with the oil extracts, so its use as housekeeping gene was considered appropriate for the present study. The pS2 mix was prepared by mixing 12.5 µl iScript SYBR Green supermix, 1 µl forward primer pS2 (CATCGACGTCCCTCCAGAAGAG; 10 µM), 1 µl reverse primer pS2 (CTCTGGGACTAATCACCGTGCTG; 10 µM), and 0.5 µl RNAse-free water. The β-actin mix contained 12.5 µl iScript SYBR green supermix, 1 µl forward primer β-actin (TTGTTACAGGAAGTCCCTTGCC; 10 µM), 1 µl reverse primer β-actin (ATGCTATCACCTCCCCTGTGTG; 10 µM), and 5.5 µl RNAse-free water. The RT-PCR was performed with a Bio-Rad MyIQTrade; single-color real-time PCR detection system. The pS2 gene transcription was finally calculated as percentage relative to the solvent control.

Data analysis

The responses induced by the oils and standards in the ER-U2OS-Luc assays were analyzed using nonlinear regression in GraphPad 4 (Prism 4; GraphPad Software). Data were fit according to a sigmoid dose-response curve with variable slope. In case of the combination experiments, predicted responses were calculated based on a multiplicative response addition approach, according to the formula presented below. The assumption for the predicted response is that the combined effect of two substances with a different toxic mode of action will be such that one substance will affect only the proportion of cells that is not affected by the first substance [22,23]:

$$predicted\ response = (1 - (1 - EEF_{EC50}) \times (1 - EEF_{oil}))$$

in which:

$$EEF_{EC50} = \frac{Effect\ EC_{50}E2}{Effect\ EC_{100}E2}$$

$$EEF_{oil} = \frac{Effect\ oil}{Effect\ EC_{100}E2}$$

The measured responses can be equal to, lower than, or higher than the predicted ones, thereby indicating additivity, antagonism, and synergism, respectively. Measured responses were considered significantly different from the predicted responses if $p < 0.05$ (one sample *t* test).

Results and Discussion

Estrogenic activity of oils in the absence of E2

To determine the estrogenicity of the oils, human osteosarcoma cells transfected with either ERα (ERα-U2OS-Luc) or the ERβ (ERβ-U2OS-Luc) were exposed to different oil concentrations (0.25-100 mg/L). In the ERα-U2OS-Luc assay, no estrogenic responses were measured for Hollmix crude (HMC) at any concentration tested (Fig. 4.1A and Table 4.1). In case of Arabian, Romanian, and Oseberg crude oil (AC, RC, and OC, respectively), estrogenic responses amounting to approximately 17% of the maximum responses of 17-β estradiol (E2max) were measured at a concentration of 50 mg/L (Fig. 4.1A and Table 4.1). The refined products gave more diverse responses, ranging from no effect in the case of gasoline (GO) and kerosene (KS) to approximately 90% of the E2max in case of 50 mg/L Bunker 500 (B500; Fig. 4.1B and Table 4.1). In the ERβ-U2OS-Luc assay, the maximum responses of cells to increasing concentrations of the crude oils AC, RC, OC, and HMC ranged from approximately 20% of the E2max for HMC at 33 mg/L to 44% of the E2max for OC at 25 mg/L (Fig. 4.1C and Table 4.1). The maximum responses for the refined products ranged from 8% for GO and KS at 25 mg/L to approximately 65% of the E2max for distillate marine grade A (DMA) and B500 at 33 mg/L (Fig. 4.1D and Table 4.1).

Literature on estrogenic effects of oils in mammalian cells is scarce. So far, either only weak estrogenic responses (for two heavy oils [6]) or no effects (in case of two crude oils and a clarified slurry oil [8]) have been observed in the human metastatic mammary adenocarcinoma cell line MCF-7. The difference between the present results and the above-mentioned ones presumably can be explained by the differences in composition (the presence or concentrations of the agonists) of the oils tested. Oils extracted from different wells have different chemical compositions, which will finally determine their toxicity [24].

This is also illustrated within the present results; the estrogenic responses measured for HMC were approximately two to six times lower than those measured for the other crudes.

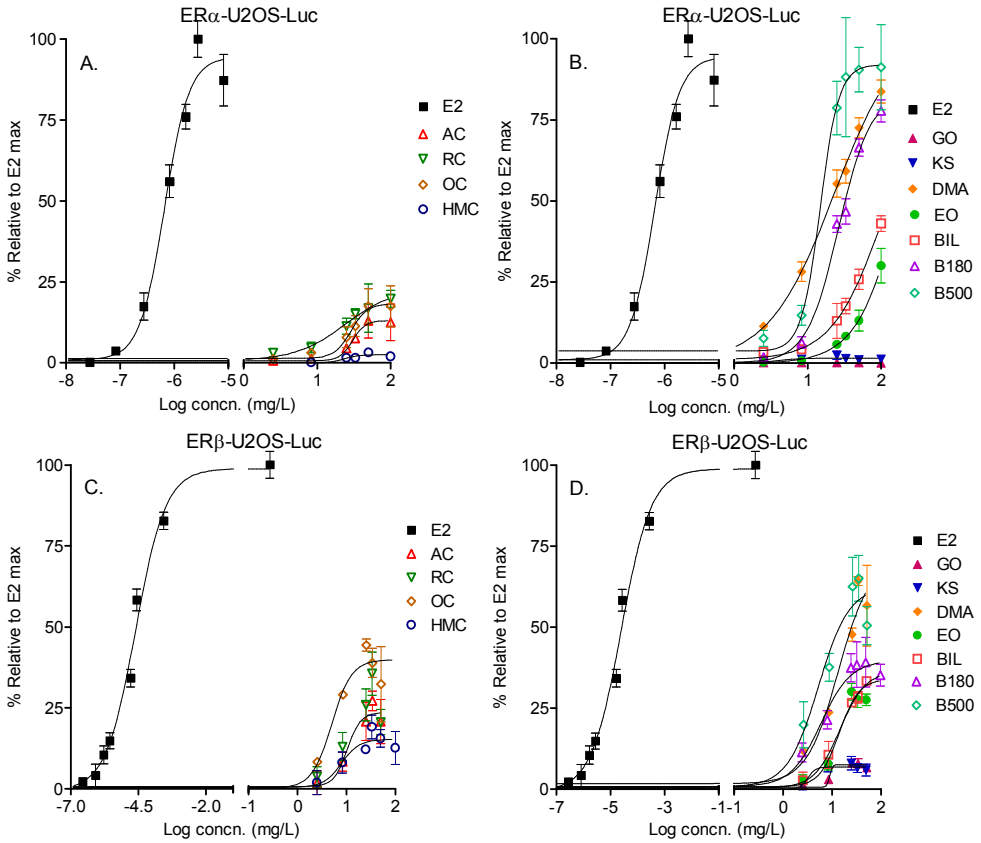

Figure 4.1: Estrogenic effects of different concentrations of crude oils (Arabian crude [AC], Romanian crude [RC], Oseburg crude [OC], and Hollmix crude [HMC]) and refined petroleum products (gasoline [GO], kerosene [KS], distillate marine grade A [DMA], engine oil [EO], bilge oil [BIL], Bunker 180 [B180], and Bunker 500 [B500]) in the estrogen receptor alpha osteosarcoma cell assay, expressing luciferase protein (ERα-U2OS-Luc; **A** and **B**) and in the estrogen receptor beta osteosarcoma cell assay, expressing luciferase protein (ERβ-U2OS-Luc; **C** and **D**). Data (± standard deviation; *n* = 3) are expressed as percentage luciferase formation relative to the maximum luciferase production induced by the 17-β estradiol (E2) standard.

On the whole, in the present study, no difference in the estrogenic efficacy of oils mediated via either the ERα or the ERβ were observed. Exceptions were OC and HMC, which were stronger inducers in the ERβ-U2OS-Luc than in the ERα-U2OS-Luc assay and DMA, and

Bunker 180 (B180), and B500, which were more active in the ERα-U2OS-Luc than in the ERβ-U2OS-Luc assay (see Fig. 4.1). The difference in the ligand binding domain between these two receptors (there is only 56% similarity) may imply that ligands from oils can bind with different affinities to the receptors. Upon binding to the receptors, these oil constituents will induce specific conformational changes in the receptors. For example, it has been demonstrated that in ERα a hydrophobic groove is formed in response to agonist binding, which is important for the binding of the p160 coactivator proteins [25]. Furthermore, the different recruitment of specific coactivators on ERα by different ligands has been related to the full or partial agonistic character of the ligands [25]. In the case of ERβ, the failure of recruiting p160 coactivators by ligands was correlated with the absence of agonist activity [25].

Table 4.1: Estrogenic potencies of 11 crude and refined petroleum products relative to 17-β estradiol (E2), following 24 h of exposure in the estrogen receptor alpha osteosarcoma cell assay expressing luciferase protein (ERα-U2OS-Luc) and in the estrogen receptor beta osteosarcoma cell assay expressing luciferase protein (ERβ-U2OS-Luc).

	ERα						ERβ					
Standard/oil	LEC[a] (mg/L)	Relative potency[b] (LEC)	EC50[c] (mg/L)	IEQ_{E2}[d] (µg/g)	Max response[e] (%)	Conc. max[f] (mg/L)	LEC[a] (mg/L)	Relative potency[b] (LEC)	EC50[c] (mg/L)	IEQ_{E2}[d] (µg/g)	Max response[e] (%)	Conc. max[f] (mg/L)
17β-Estradiol	3.0E-08	1	6.6E-07	1.0E+06	100 ± 5.6	2.72E-06	2.3E-07	1	2.5E-05	1.0E+06	100 ± 4.2	0.2700
Refined products												
Gasoline	NC	NC	NC	NC	0.14 ± 0.3	25	4.9E+00	4.7E-08	8.95	2.8E+00	8.11 ± 1.7	25
Kerosene	4.2E+00	7.2E-09	NC	NC	2.36 ± 1.3	25	9.3E+00	2.5E-08	3.19	7.8E+00	8.04 ± 3.6	25
Distillate marine grade A	2.1E+00	1.4E-08	23.3	2.8E-02	83.7 ± 3.5	100	3.5E-01	6.6E-07	13.5	1.9E+00	64.2 ± 2.6	33
Engine oil	4.9E+00	6.1E-09	NC	NC	29.9 ± 5.4	100	4.5E+00	5.1E-08	14.0	1.8E+00	30.1 ± 4.4	25
Bilge oil	3.9E-01	7.7E-08	NC	NC	42.9 ± 2.4	100	4.4E+00	5.3E-08	14.5	1.7E+00	34.6 ± 10	100
Bunker 180	5.0E-01	6.1E-08	26.5	2.5E-02	77.7 ± 3.4	100	2.2E-01	1.1E-06	5.91	4.2E+00	39.1 ± 13	50
Bunker 500	2.5E-01	1.2E-07	14.8	4.4E-02	90.4 ± 6.8	50	1.2E+00	1.9E-07	5.25	4.8E+00	65.1 ± 7.1	33
Crude oils												
Arabian crude	1.3E+00	2.3E-08	30.5	2.2E-02	13.0 ± 5.3	50	1.7E+01	1.3E-08	10.4	2.4E+00	27.2 ± 5.2	33
Romanian crude	9.3E-01	3.2E-08	24.4	2.7E-02	16.9 ± 7.4	50	2.3E+00	1.0E-07	NC	NC	35.5 ± 12	33
Oseberg crude	2.0E+00	1.5E-08	28.8	2.3E-02	17.5 ± 5.3	50	3.0E-01	7.7E-07	5.03	5.0E+00	44.4 ± 1.9	25
Hollmix crude	1.1E+01	2.8E-09	22.9	2.9E-02	3.23 ± 0.3	50	5.5E+00	4.2E-08	7.91	3.2E+00	19.2 ± 6.2	33

[a] Lowest-effect concentration; test concentration at which first effects are observed.
[b] Ratio of the dose of estradiol and that of oil needed to achieve the LEC.
[c] Concentration at which 50% of maximum luciferase induction is reached.
[d] Estradiol induction equivalents; number of micrograms of E2 present in 1 g of oil
[e] Percentage luciferase induction (± standard deviation) calculated as the maximum response of oil relative to the maximum response for estradiol.
[f] Concentration oil at which the maximum response is reached.
NC: not calculated; maximum effect not reached.

Interestingly, the results obtained in the present study with mammalian cells clearly differ from our previous results obtained with a yeast stably transfected with either human ERα or ERβ and yeast enhanced green fluorescent protein [10]. In the ERα yeast assay, none of the oils induced responses, whereas, in the ERβ yeast assay, almost all of the oils had estrogenic

activities [10], including supermaximal ones. Differences in absorption, transport, and metabolism of the agonists and the presence of coactivator proteins may explain the differences between the results obtained with mammalian versus yeast assays. Also, mammalian assays allow the detection of functional (receptor-mediated) effects that are induced indirectly, e.g., by interaction with nuclear factors, effects on receptor levels, or interaction with net influx or metabolism in the cells, all being effects with in vivo relevance as well. In addition, in a meta-analysis of in vitro reporter gene assays it was demonstrated that the number of EREs and the nature of the promoters could also play a role [26]. Importantly, the differences between different assays stress the need for testing samples in more than one assay when trying to identify environmental estrogens or when labeling or classifying compounds. Different assays should be regarded as complementary, and their application may help in identifying the real mechanism of action of xenoestrogens [27]. In the end, inevitably estrogenic responses measured in vitro should be confirmed in in vivo studies [28].

Estrogenic activity of oils in combination with E2

Any antiestrogenic, additive, or synergistic effects of oils in the ER-U2OS-Luc assays were assessed by exposing the cells to different concentrations of the oils (0.25-100 mg/L) in combination with the EC50 of E2 (i.e., 3 and 100 pM for the ERα- and ERβ-U2OS-Luc assay, respectively). In the ERα-U2OS-Luc assay, the measured responses for KS at 25, 33, and 50 mg/L were significantly lower than the predicted ones ($p < 0.05$), thereby indicating antiestrogenic effects (Fig. 4.2). In contrast, the measured responses were significantly higher than predicted ones, in the case of OC at 100 mg/L, B180 at all concentrations, and B500 at all concentrations except for 50 mg/L, possibly indicating synergistic effects (Fig. 4.2A and B). No effects were measured in the case of GO and HMC, but for the remaining oils, the measured responses were not significantly different from the predicted ones, indicating additivity (Fig. 4.2A and B). In the ERβ-U2OS-Luc assay, no antiestrogenic effects were measured. However, higher-than-predicted, and thus possibly synergistic, effects were observed for all oils except for AC, albeit only at specific concentrations (100 mg/L for GO; 8.3 and 100 mg/L for KS; 25 mg/L for DMA; 50 mg/L for EO; 50 and 100 mg/L for BIL; 33, 50, and 100 mg/L for RC; 2.5, 8.3, 25, and 100 mg/L for OC; 2.5, 8.3, and 100 mg/L for HMC; 8.3, 50, and 100 mg/L for B180; and 2.5, 8.3, 33, and 50 mg/L for B500; Fig. 4.2C and D). For the remaining concentrations, the measured effects were not significantly different from the predicted ones, indicating additivity. The fact that synergism is not observed at all concentrations is difficult to explain, but might suggest a low statistical power of the model used or interactions among the chemicals present in the mixture affecting different pathways in the cells [29,30].

The current observation that almost all of the oils displayed synergistic estrogenic effects is noteworthy, insofar as such effects have to date been reported in the literature only once, for a mixture of increasing concentrations of E2 and subeffective concentrations of *cis-trans*-tamoxifen and 4-hydroxytamoxifen in a yeast stably transfected with human ERα, [31]. Effects on receptor phosphorylation and stabilizing effects on the receptor half-life, DNA-receptor complex formation, and mRNA are some of the explanations for these effects as provided by the authors. These explanations may apply to the present results as well, although they obviously remain strictly hypothetical.

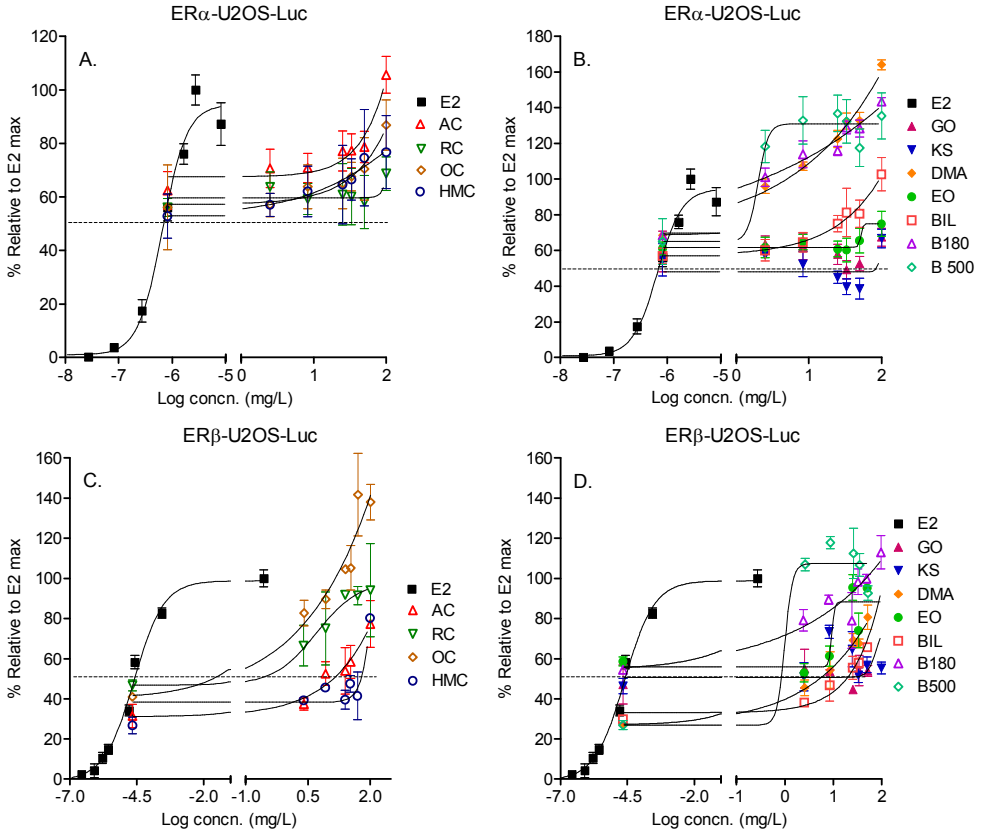

Figure 4.2: Estrogenic effects of different concentrations (0.25-100 mg/L) of crude oils (Arabian crude [AC], Romanian crude [RC], Oseburg crude [OC], and Hollmix crude [HMC]) and refined petroleum products (gasoline [GO], kerosene [KS], distillate marine grade A [DMA], engine oil [EO], bilge oil [BIL], Bunker 180 [B180], and Bunker 500 [B500]) on coexposure to 3 pM (i.e., 8.17 x 10^{-7} mg/L; 50% effect concentration [EC50]) of 17-β estradiol (E2) in the estrogen receptor alpha osteosarcoma cell assay expressing luciferase protein (**A** and **B**) or 100 pM (i.e., 2.71 x 10^{-5} mg/L; EC50) of E2 in the estrogen receptor beta osteosarcoma cell assay expressing luciferase protein (**C** and **D**). Data (± standard deviation; *n* = 3) are expressed as percentage luciferase formation relative to the maximum luciferase production induced by the E2 standard.

The present study does suggest that exposure to the oils at relatively low concentrations might lead to ERα or ERβ activation in the tissues in which these receptors are specifically expressed, such as breast, uterus, and bones, and thereby might influence cell growth and differentiation [32]. This issue will be discussed further below.

E2-like potency of oils

To characterize the oils further, the relative estrogenic potencies of the oils were calculated. Because no full dose-response curves were obtained for GO, KS, EO, and BIL in the ERα assay and for RC in the ERβ assay, an EC50 could not be calculated for all oils. Therefore, the potencies were based on the lowest-effect concentration (LEC) that could be derived in all cases. The resulting potencies in the ERα-U2OS-Luc assay for BIL, B180, and B500 were approximately seven orders of magnitude lower than that of E2 (Table 4.1), whereas for the other oils they were approximately eight to nine orders of magnitude lower. In the ERβ-U2OS-Luc assay, the most potent oils were DMA, B180, and OC, having relative potencies of about six orders of magnitude lower than that of E2, whereas for the other oils the potencies were seven to eight orders of magnitude lower than that of E2. The potencies revealed in the present study generally are of the same order of magnitude as the ones previously determined with the yeast assays [10], although in specific cases the relative estrogenic potencies obtained by the yeast assays were higher. For instance, for B180 and AC, a two orders of magnitude higher potency was derived with the yeast assays. These results suggest that yeast assays are a valuable screening tool [10]. However, the differences between the calculated relative potencies again demonstrate that one should not have implicit faith in the results of a single assay. We do not have a conclusive explanation for the lower relative potencies obtained for some of the oils with the currently applied mammalian assays, because a number of differences between the yeast and the U2OS-Luc assays might contribute (for example, differences in metabolic capacity, cell membrane structure, and bioavailability of agonists resulting from differences in medium composition).

Although the results of two independent in vitro assays (the present study and the previous work on yeast) indicated the estrogenic potencies of the oils studied, parameters such as bioaccumulation, pharmacokinetics, and cross-talk with other mechanisms should be taken into account when extrapolating the results to the in vivo situation. Because these parameters are not included in in vitro assays, our data should be considered an indication of the transcriptional activation potential of certain oils that might lead to effects in hormone-sensitive tissues. Examples of such effects are discussed below.

MCF-7 cell proliferation and pS2 gene expression

The binding of an estrogenic compound to the ERα in human mammary cells will lead to the induction of cell proliferation, which has been linked in the literature to the formation of cancer [33]. The estrogen-responsive human breast cancer cell line MCF-7, naturally

expressing the ERα, was used in the present study to investigate whether the oils could elicit an estrogen-dependent biochemical response in a whole cell, such as induction of cell proliferation and an increase of pS2 gene expression. Based on the estrogenic activities observed in the present study with ER-U2OS-Luc cells and in our previous study with yeast [10], DMA was selected as testing substance to study these responses. The oil produced a clear dose-response relationship in the MCF-7 assay: cell proliferation increased with oil concentration, with a maximum effect of approximately 70% of the maximum proliferation observed for E2 (Fig. 4.3). The lowest DMA concentration at which first proliferative effects were observed (25 mg/L) was subsequently chosen to study effects on pS2 gene transcription. After 5 d of exposure, the pS2 mRNA expression in MCF-7 cells was significantly induced by a factor of two compared with the 0.5% solvent control (Fig. 4.4). Note that an increase in gene expression of >1.5-fold is generally considered a cut-off value for significant induction above background [34,35]. To the best of our knowledge, this is the first study to describe the effects of oil on MCF-7 cell proliferation and gene expression. The results should however be considered preliminary; for establishing a full estrogenic profile, the remaining oils should be tested as well, preferably at multiple concentrations.

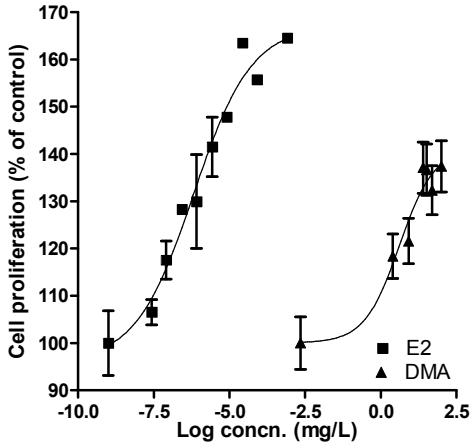

Figure 4.3: Cell proliferation in MCF-7 human mammary carcinoma cells after exposure for 5 days to different concentrations (2.25-100 mg/L) of distillate marine grade A (DMA) oil. Data (±standard deviation; *n* = 3) are expressed as percentage relative to the ethanol control.

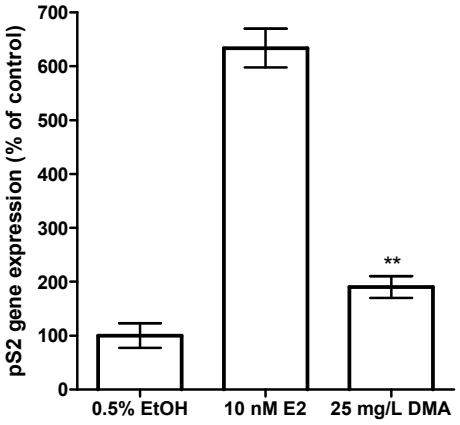

Figure 4.4: Induction of pS2 gene transcription in MCF-7 human mammary carcinoma cells after exposure for 5 d to the 0.5% ethanol control, 2.7 × 10^{-3} mg/L of 17-β estradiol (E2), and 25 mg/L of distillate marine grade A (DMA) oil. Data (±standard deviation; *n* = 3) are expressed as percentage pS2 gene transcription relative to the ethanol control. **Significantly different from control ($p < 0.005$).

Environmental relevance

The effects observed in the present study add to the existing literature on specific effects of oils on biota. Several studies have shown that oils contain compounds that can cause estrogenic responses in vitro [6,10,11]. In addition, effluents from offshore and land-based oil production have been demonstrated to contain estrogenic compounds capable of displacing E2 from plasma steroid hormone-binding globulin, thus potentially being able to modulate the endocrine system [36]. Furthermore, elevated P4501A1 mRNA in American mink resulting from ingestion of low concentrations of Bunker C oil and effects of heavy oil on the early life stages of spotted halibut have been observed [2,37]. Such effects of oil can be detected in wildlife even many years after the pollution occurred [38].

The effects demonstrated in the present study were observed at concentrations that can be considered environmentally relevant. For instance, concentrations of approximately 8 to 15 mg oil/L treated refinery effluent have been reported [39], and water from North Sea production platforms contained about 30 to 60 mg oil/L [40]. Concentrations resulting from oil spills, such as the recent Deepwater Horizon disaster in the Gulf of Mexico, may be expected to be even higher, although values may be site-specific and will depend on factors such as the type (solubility) of the oil. Oil pollution may adversely affect not only nature but also social and economic functions of ecosystems, such as fisheries, aquaculture and tourism. Predicting the final risks of oils for humans and the environment is challenging, insofar as they will depend among other things on the bioavailability and bioaccumulation of the active chemicals present in oils, the sensitivity of the species, the weathering state of the oil, and the combination with any oil-spill chemicals used. Moreover, oil is a complex mixture, and the overall effects will be caused by the sum of all possible effects of all oil constituents, whose concentrations may vary between different oils. However, as far as the risk assessment of oils is concerned, it would be advisable not to focus on nonspecific, narcotic effects only but to consider specific, more subtle effects as well, such as the estrogenic ones described in the present paper.

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Chapter 5

Effect-directed assessment of the bioaccumulation potential and chemical nature of A*h* receptor agonists in crude and refined oils.

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To be submitted

Abstract

Recent studies have indicated that in addition to narcotic chemicals, crude oils and refined petroleum products also contain compounds with a specific mode of action, such as dioxin- and PAH-like aryl hydrocarbon receptor (AhR) agonists. The risks of these toxic compounds to organisms will depend on internal exposure levels, as driven by the chemicals' bioaccumulation potential. Information on this potential however is lacking, as the chemicals' identity is mostly unknown. In this study, bioaccumulation experiments first of all demonstrated that the agonists are taken up from oil-spiked sediments and persist in aquatic worms for at least several weeks. Chemical (SARA) fractionations of eight pure oils, followed by effect-directed analyses using *in vitro* reporter gene assays revealed that the agonists predominantly are aromatic and resin-like chemicals, some of which are easily metabolized, while others are resistant to biotransformation *in vitro*. HPLC-assisted hydrophobicity profiling indicated that the active chemicals had a high to extremely high bioaccumulation potential, with estimated logK_{ow} values of 4 to >10, with the majority of activity being assigned to compounds with logK_{ow} of 5-8. These compounds were present mainly in the mid to high boiling point fractions of the oils, which are usually not being considered (the most) toxic in current risk assessment. The fractionations further revealed considerable oil and fraction-dependent complex mixture effects (antagonism) in pure oils and SARA fractions. The results of this study clearly demonstrate that crude oils and refined petroleum products contain numerous compounds that can activate the AhR and because of the known persistence and extremely high bioaccumulation they are potential PBT candidates. Many chemicals were identified by GC-MS, but the responsible individual compounds are not easily identified in the complex mixtures of thousands of compounds. Because this obstructs a classical PBT risk assessment, our results advocate the need for a serious attempt to develop a more advanced risk assessment approach.

Introduction

Current risk assessment of oils (petroleum hydrocarbons) is mostly based on acute toxicity and considers narcosis (non-specific effects) as the sole mode of action [1,2]. Several epidemiological and *in vivo* and *in vitro* experimental studies, however, have suggested or demonstrated potential specific, chronic effects of petroleum hydrocarbons. For example, humans living in the vicinity of petroleum explorations have a higher incidence of cancer [3]. In wildlife, chronic oral exposure of mink to heavy bunker oil resulted in systemic effects, such as altered hepatic metabolism, decreased number of circulating leucocytes, and altered adrenal physiology [4]. In fish and birds, sublethal, chronic exposure to petroleum was associated with reproduction impairment and altered development at early life stages [5]. Furthermore, several *in vitro* studies have shown that crude oils, refined petroleum products, and offshore produced waters contain chemicals that can associate with the aryl

hydrocarbon, estrogen, and androgen receptors, thereby possibly being able to cause a suite of chronic, dioxin-like and endocrine disrupting effects [6-14]. Thanks to this *in vitro* work, important advances in the hazard identification and characterization of oils have been achieved. Nevertheless, for proper risk assessment, knowledge on exposure is also required. Although oils are ubiquitous in the environment, risks are defined by the actual internal exposure of organisms [15], which in case of petroleum hydrocarbons is firstly determined by the potential of the compounds to bioaccumulate. The bioaccumulation potential of petroleum hydrocarbons is expected to depend mainly on their hydrophobicity, since most of the compounds are nonionic, apolar organic compounds. However, there is very little knowledge available on this issue, as the identity of the petrochemicals causing specific effects is mostly unknown.

Recently, by applying effect-directed analyses (EDA), Thomas et al. [16] revealed that petrogenic naphthenic acids were the major compounds causing the estrogenic and anti-androgenic activities in offshore produced water tested in *in vitro* yeast assays. Naphthenic acids are highly water soluble, polar compounds however, that hardly bioconcentrate in fish, with bioconcentration factors of only about 2 [17]. Therefore, internal concentrations of these compounds in organisms and hence the actual risks *in vivo* at environmentally-relevant concentrations are expected to be low. With respect to the other type of potential specific chronic effects reported for oils, i.e., A*h* receptor (AhR)-mediated effects, there is hardly any information on the identity and the bioaccumulation potential of the causative petroleum hydrocarbons [18]. Such knowledge is crucial, though, if chronic effects were to be included in risk assessment of oils.

The objective of the present study therefore was to assess the bioaccumulation potential of the AhR agonists in a series of 8 oils for which previously AhR-mediated effects were reported [8]. First, in order to investigate whether the AhR agonists actually bioaccumulate *in vivo*, bioaccumulation experiments were performed with aquatic worms. The organisms were exposed for 4 weeks to oil-spiked sediments, after which they were extracted and the extracts were tested in the chemically-activated fluorescent gene expression bioassay (CAFLUX) for AhR activation. Second, the pure oils were fractionated into so-called SARA (saturates, aromatics, resins, and asphaltenes) fractions, of which the two active ones were further fractionated by reversed-phase HPLC into 9 sub-fractions with increasing hydrophobicity (octanol-water partition coefficient [K_{ow}]) ranges, reflecting different bioaccumulation potentials. All fractions were then tested in a dioxin receptor-mediated luciferase (DR-Luc) assay in order to link AhR-mediated effects to bioaccumulation potential. Finally, the active fractions were analyzed by gas chromatography with flame ionization detection (GC-FID) and mass spectrometry (GC-MS) in an attempt to chemically characterize the fractions in more detail and to validate the HPLC-assisted hydrophobicity profiling.

Materials and Methods

Chemicals

Solvents used were acetone, n-hexane (Pestican grade; Lab Scan, Ireland), dichloromethane, methanol (HPLC grade; Lab Scan), ethanol (LiChrosolv grade; Merck, Germany), and toluene (Spectranal grade; Riedel-de Haën, Germany). Aromatic and resin compounds used as calibration compounds for HPLC fractionations (all > 97% pure) were furan, 2-methylfuran, dibenzofuran, pyrrole, N-methylpyrrole, benzo[*b*]pyrrole, carbazole, 9-ethylcarbazole, thiophene, benzothiophene, pyridine, 2-ethylpyridine, 5-ethyl-2-methylpyridine, benzo[*b*]quinoline (all from Acros; Geel, Belgium); benzofuran, 3-methylthiophene, 2-propylthiophene, 3-octylthiophene, benzo[*b*]naphthol[*2,1-d*]thiophene, 4-methylquinoline, phenanthrene, pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*e*]pyrene (Aldrich; Milwaukee, USA); naphthalene, dibenzothiophene, coronene (Fluka; Buchs, Germany); acenaphthene, chrysene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene (Dr. Ehrenstorfer; Augsburg, Germany); anthracene (Sigma; St. Louis, USA); fluoranthene, indeno[*123-cd*]pyrene, and benzo[*ghi*]perylene (AccuStandard; New Haven, USA). Other chemicals used were aluminum oxide (90 active neutral; Merck, Germany; and Super I; MP Biomedicals, Eschwege, Germany) and anhydrous sodium sulfate (analytical grade; Merck).

Organisms and sediment

Organisms used for the bioaccumulation experiment were aquatic oligochaete worms (*Lumbriculus variegatus*), which were reared in the laboratory as described before [19]. Prior to the experiments, organisms were gut-purged for 16 hours under gently flowing tap water.

The sediment was sampled from a shallow, pristine pond near Cothen, the Netherlands. It was passed through a 1 mm sieve, homogenized, and stored at 4 °C until use. The presence of any interfering native AhR agonists was tested as follows. Approximately 1 g of sediment was Soxhlet-extracted overnight with hexane/acetone (3:1). The extract was concentrated to 1 mL using a modified Kuderna-Danish apparatus and nitrogen, and cleaned-up through 4 g of aluminum oxide (Super I) with 30 mL of hexane. The eluate was concentrated to 0.5 mL, 1.5 mL of ethanol was added, and the resulting extract was concentrated under nitrogen to 1.0 mL. This sample was tested in the CAFLUX assay according to the method described below.

Oils

The oils tested included four refined products (Distillate Marine grade A [DMA], Bilge oil [BIL], Bunker 180 [B180], and Bunker 500 [B500]) and four crude oils (Arabian crude [AC],

Romanian crude [RC], Hollmix crude [HMC], and Oseberg crude [OC]). Applications, sources, and/or suppliers of the oils are presented in Table A1.

Bioaccumulation experiment

Eight 1 L brown jars were filled with approximately 700 mL of wet sediment each and a large metal stir bar was added. The jars were placed on magnetic stirrers and while intensively stirring, the sediments were spiked with either BIL or B180 oil, to obtain nominal oil concentrations of 0, 500, 1250, or 3000 mg/kg dw for BIL; and 0, 500, 1000, or 1500 mg/kg dw for B180. Pilot experiments had indicated that these concentrations were not lethal to *Lumbriculus variegatus*. The jars with spiked sediments were placed on a roller coach for 4 weeks. After this homogenization period, the content of each jar was divided over 3 of 1L jars. The resulting jars contained a layer of about 3 cm of (consolidated) sediment, on top of which about 800 mL of tap water was placed. To each of these systems, 5 g (wet weight) of worms was added, after which they were left for 4 weeks at 20 °C under gentle aeration and without the addition of food. At the end of the exposure time, the worms were manually separated from the sediments, placed in clean water for 6 hours to allow gut purging, frozen at -20 °C, and freeze-dried overnight. The resulting samples were homogenized and about 40 mg of each sample was Soxhlet-extracted and cleaned-up as described above. The final extracts in ethanol were tested in the CAFLUX assay according to the method described below. Lipid contents of the worms were determined gravimetrically, after Soxhlet-extracting the worms as described above.

SARA fractionations

All oils were fractionated into saturates, aromatics, resins, and asphaltenes as described in detail in the Appendix. Briefly, asphaltenes were first precipitated by washing with n-hexane, according to methods described by Aske et al. and Hannisdal et al. [20,21], after which they were dried under nitrogen and dissolved in toluene. The pooled hexane phase was concentrated to 1 mL using a modified Kurdena-Danish apparatus and nitrogen, and separated into the remaining 3 fractions by open column chromatography according to EPA guideline 3611 B [22]. Additionally, after the last elution step, the column material was extracted with toluene (yielding a 'column extract' as fifth fraction), in order to check for AhR agonists remaining on the column. Finally, all fractions were exchanged to or diluted in ethanol.

HPLC fractionations

The aromatic and resin fractions which contained the majority of AhR agonists (see Results and Discussion) were further separated into 9 sub-fractions, using reversed phase HPLC. Retention time (sampling) windows of the sub-fractions were chosen based on a

calibration curve of retention time against $\log K_{ow}$ (Figure A1) of a series of 21 resins (four furans, five pyrroles, seven thiophenes, and five pyridines) and 16 polycyclic aromatic hydrocarbons (PAHs), known to occur in oils. The time windows chosen for collecting the consecutive 9 sub-fractions corresponded to the following $\log K_{ow}$ ranges: 0-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, and 9-10.5 (see Appendix). Both calibration compounds and oil aromatic and resin fractions were dissolved in ethanol of which 100 μL was injected on an Alltima C_{18} column (150 x 7 mm, 5 μm particle size), with a Phenomenex C_{18} guard column (4.0 x 3.0 mm), using water/methanol as mobile phase. Water/methanol fractions (about 12 mL) were collected, diluted with Millipore water, and extracted with hexane, which was concentrated and exchanged to 1.0 mL of ethanol. More details on the procedure are provided in the Appendix.

In vitro assays

Extracts of worms and sediments were tested in the CAFLUX assay [23], as described in the Appendix. In short, 1 μL of extract was added to the wells of a 96 well plate, containing a mouse hepatoma cell line (H1G1), stably expressing a fluorescent AhR-activated reporter gene construct. After 24 h of exposure, the fluorescence was measured on a FLUOstar (BMG, Labtechnologies) at an excitation and emission wavelength of 485 nm and 510 nm, respectively. SARA and HPLC fractions were tested in the DR-Luc assay, as described previously [8] and in the Appendix. To distinguish between persistent and easily-metabolizable compounds, exposure time was both 6 and 24 h [8].

The CAFLUX and DR-Luc assay are based on the same principle that specific chemicals bind to and activate the AhR, resulting in enhanced transcription and translation of the reporter gene. In addition, cytochrome P450 1A is induced, which can metabolize e.g. most PAHs. As the reporter of the DR-Luc assay, luciferase, is only stable for a few hours, a distinction can be made between metabolically labile compounds (such as PAHs) that disappear after 6-12 hrs and persistent AhR ligands that continue to be present and induce the luciferase production [24]. The CAFLUX reporter is more stable and therefore an integrated total AhR induction is indicated during the full exposure period [25].

GC analyses

GC-FID analyses of all HPLC sub-fractions in ethanol were performed by on-column injection of 1 μL samples as described previously [19,26]. In order to be able to relate the results to boiling point fractions, an alkane standard (containing all even-numbered alkanes between C_{10} and C_{40} in ethanol) was analysed several times.

GC-MS analyses of 1 μL of the sub-fractions yielding a significant response in the DR-Luc assay were performed on a Thermo Trace GC Ultra (DSQ quadrupole), equipped with a TriPlus autosampler and a Varian Factor Four capillary column (VF-5ms; 30mx0.25mm, 0.25 df), while operating in the full scan mode (m/z 70-500). The NIST MS Search v 2.0 d library

was used to identify separate peaks where possible and additionally all chromatograms were scanned for these and other known literature-derived petrogenic aromatics and resins (Tables A2- A3).

Data analysis

Data resulting from the DR-Luc assay were analyzed as described in [8], by expressing the sample responses as percentage relative to the maximum response of the assay's positive standard (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] for 24 h exposures and benzo[*a*]pyrene [BaP] for 6 h exposures). In case of the bioaccumulation experiment, sample responses from the CAFLUX assay were interpolated in the dose-response curve of TCDD and expressed as mg TCDD equivalents (TEOs), normalized to worm lipid or sediment organic carbon content.

Results and Discussion

Bioaccumulation of AhR agonists in worms

In Figure 5.1, CAFLUX assay responses to extracts of worms exposed to sediment containing different concentrations of B180 oil are presented. Responses to extracts of worms exposed to BIL oil-containing sediments are presented in Figure A2. Both figures demonstrate that the response (mg TEQ/kg worm lipid) goes up with increasing oil concentration in the sediment. As the oil concentration was the only variable in the experiment, this demonstrates that the oils contain bioaccumulative AhR agonists. Moreover, it suggests the agonists are relatively persistent, since the contact time with the (non-sterilized) sediment was several weeks. As in the CAFLUX assay the reporter is stable, the responses presented in Figures 5.1 and A2 could be induced by both labile and more stable AhR ligands. The chemical nature of the agonists is discussed in more detail below.

Figures 5.1 and A2 indicate that the AhR agonists prefer animal lipids over sediment organic carbon. The agonist concentration in lipids after all is about 3 times higher than in the sediment organic carbon, which translates to a biota-to-sediment accumulation factor (BSAF) of approximately 3. This is higher than the recently observed BSAFs for oil in a series of field sediments (being always (much) less than 2; [19]). This discrepancy may be explained by the fact that the present data are based on freshly spiked sediments in which the oils will be more bioavailable than in aged sediments and soils.

Although also fouling of the worms with oil could explain the results in Figures 5.1 and A2, this artifact is not likely for the following reasons. First, the experiments were performed in the same manner as in [19], in which it was demonstrated that fouling was highly unlikely, based on chromatographic profiles of pure oils and worm extracts. Second, in the present experiment, the sediments were spiked to obtain concentrations below the so-called critical separate phase concentration (CSPC; [19,27]), i.e. the concentration above which oils form a

separate phase (droplets or films). In the absence of these separate phases, fouling is not possible.

Based on chemical analyses, it has been demonstrated before that oil can accumulate in benthic organisms and compounds up to C_{34} have been identified in worms exposed to oil-contaminated sediments [19]. Indirect evidence for the bioaccumulation potential of petrogenic AhR agonists has been obtained via measuring induction of EROD activity as biomarker for AhR agonist responses in organisms collected from locations near oil spills [28-30]. However, the present results provide direct evidence for the bioaccumulative nature of specific AhR agonists, i.e., compounds having a dioxin-like toxic potency, with a high affinity for biotic lipids. Hence, exposure to oil-contaminated sediments may lead to an internal dose of AhR agonists and thereby to a potential risk.

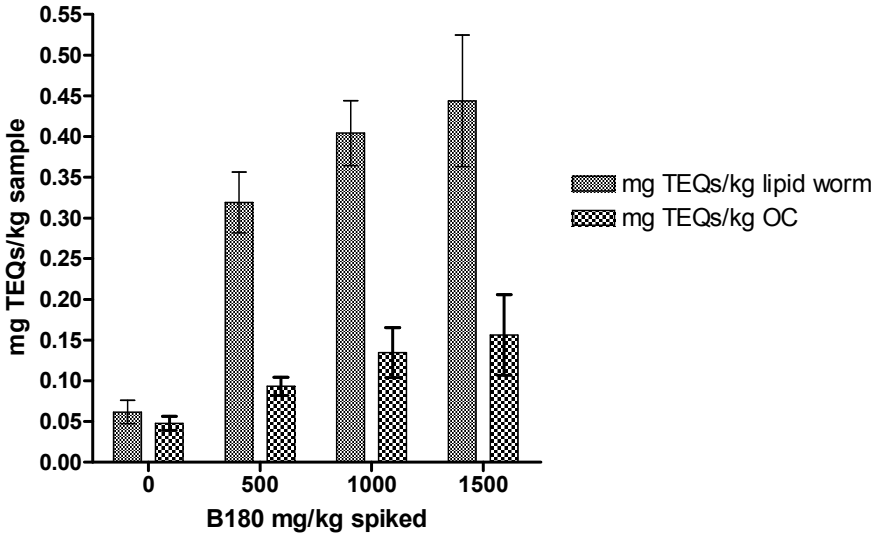

Figure 5.1. CAFLUX assay responses (mg TEQ/kg sample) to solvent extracts of worms and sediments. The worms were exposed for 4 weeks to the sediment that had been contaminated with different concentrations of Bunker 180 oil (B180; indicated on the x-axis).

Identification of AhR agonism in SARA fractions

In Figure 5.2, the DR-Luc assay responses to the different SARA fractions obtained for one selected crude oil (AC) are presented. The cells were exposed to the fractions as well as the column extract for 6h (Figure 5.2A, compared to the BaP dose-response curve) or for 24 h (Figure 5.2B, compared to the TCDD dose-response curve). The results for the other oils are presented in Figures A3 and A4. The saturates fractions did not induce any DR-Luc activity after 6 or 24 h of exposure. This was to be expected, as only aromatic compounds so far have been shown to activate the AhR [31]. Saturate (aliphatic) compounds, either straight or

branched, presumably do not fit the AhR ligand binding site. Also according to expectations, the aromatic fraction of all oils caused a clear response, both after 6 and 24 h of exposure. This indicates the presence of biotransformation-resistant (persistent) agonists, as also advocated above. Still, for instance for B180 oil, the response after 24 h (Figure A4) decreased relative to the other oils, which suggests the presence of metabolizable compounds in this oil as well. Interestingly, the more polar resin fractions induced responses after 6 h (Figure A3) as high as the aromatic fractions responses. However, after 24 h for most oils the responses induced by the resin fractions were considerably lower than those of the aromatic fraction, indicating more metabolizable compounds. However, especially for the bunker oils, the high responses of the resin fractions remained even after 24 h, indicating that oils can contain persistent AhR agonists with a more polar nature.

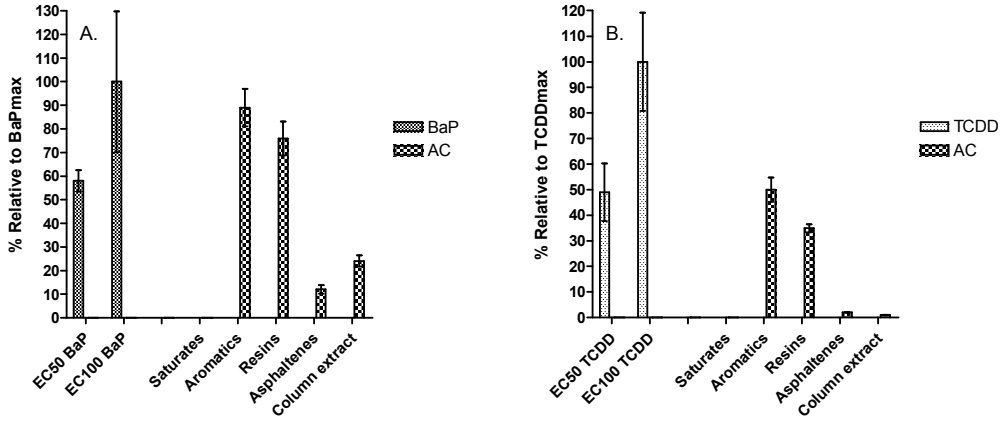

Figure 5.2. Responses to SARA (i.e. saturates, aromatics, resins, asphaltenes) and column extract fractions of Arabian Crude (AC) oil after 6 h (A) and 24 h (B) of exposure in the DR-Luc assay.

The asphaltenes of 4 of the oils surprisingly gave a clear response after 6 h as well (Figures 5.2, A3, and A4), which had disappeared, however, after 24 h. Because it is not plausible that the large asphaltenic compounds [MW > 700 g/mol [32]] can bind to the AhR and/or can be metabolized, most probably the deasphaltenation (washing the oils with hexane) did not fully remove all (aromatic or resin) agonists. Note that the completeness of the washing process was judged visually and that asphaltenes are known to be able to entrap smaller molecules inside their large molecular matrix [33,34]. Such smaller molecules in this case probably were AhR agonists that were not fully removed by hexane, but could be released from their matrix when the asphaltenes were dissolved in toluene (and later in ethanol). We therefore consider the responses induced by the asphaltenic fractions to be false positives.

Finally, as can be seen in Figures 5.2, A3, and A4, not all compounds were eluted from the columns following the EPA fractionation procedure. Considerable amounts of agonists were extracted from the column material after the final elution. The interactions between these specific petrochemicals and the aluminum oxide apparently were too strong to break by the (volumes of the) solvents applied. The fact that the compounds could be extracted with toluene suggest an aromatic nature of the compounds that, similar to the agonists retained by the asphaltenes, were easily biotransformed, as the responses had disappeared after 24 h of exposure.

Summation of the TEQs (determined after 24 h of exposure) of all fractions, including the column extract, and comparing the results to the responses observed for the unfractionated oils at 25 mg/L indicated that only 35 to 74 % of the initial activity was recovered (Table A2). Exception was BIL oil, for which the recovery measured 477 %. The low recoveries suggest that some more volatile agonists (e.g., alkylated naphthalenes) may have been lost during the concentration steps, but uncertainties in the interpolations and the resulting TEQs of the fractions may also contribute to a non-perfect mass balance. The extremely high recovery for BIL oil on the other hand can only be explained by antagonistic effects in the whole oil mixture. Petrochemicals from different SARA fractions presumably antagonize each other and by separating them this mixture effect cannot occur anymore.

From the above it appears that most AhR agonists in the oils tested were part of the aromatic and resin fractions. Hence, these fractions were further subjected to a hydrophobicity profiling procedure in order to assess the bioaccumulation potential of the agonists.

EDA of the bioaccumulation potential of petrogenic AhR agonists

Figure 5.3 shows the results of the experiment in which the HPLC fractionation-obtained aromatic and resin sub-fractions of AC oil were tested in the DR-Luc assay. The results for the other oils can be found in Figures A5-A6. The aromatic agonists were present in sub-fractions 5-9, with sub-fractions 6 and 7 being responsible for most of the activity for all oils, both after 6 and 24 h of exposure. Apparently, the aromatic agonists are persistent compounds. On the other hand, the relative contribution of separate fractions (i.e., the bar pattern in Figures 5.3, A5, and A6) generally changed with increasing exposure time, thus pointing to some labile compounds as well. Interestingly, upon 6 h of exposure, sub-fractions 6-8 of the oils AC, HMC, and B180 induced supermaximal responses. For AC and HMC oil these effects were observed for the whole oils as well, but for the total aromatic fractions the supermaximal effects did not occur. This suggests mixture effects depending on different combinations of different fractions of the oils. Sotoca et al. [35] argued that superinduction may be attributed to stabilization effects of chemicals on the luciferase enzyme half-life, while a meta-analysis showed that the cell model and construct are more important than the type of reporter used [36].

For the resin sub-fractions of most oils, the AhR activities after 6 h of exposure mainly occurred in sub-fractions 4-6, but in particular for the bunker oils the activity was also induced by sub-fractions 3 and 7-9 (Figure A5). Upon 24 h of exposure, sub-fractions 5-7 appeared the most active (Figure A6), while for the bunker oils the range of active sub-fractions was broader (4-9). In accordance with the earlier observation that the resins are less metabolically persistent than the aromatics (except those in the bunker oils), the responses after 24 h were considerably lower as compared to those after the 6 h exposures.

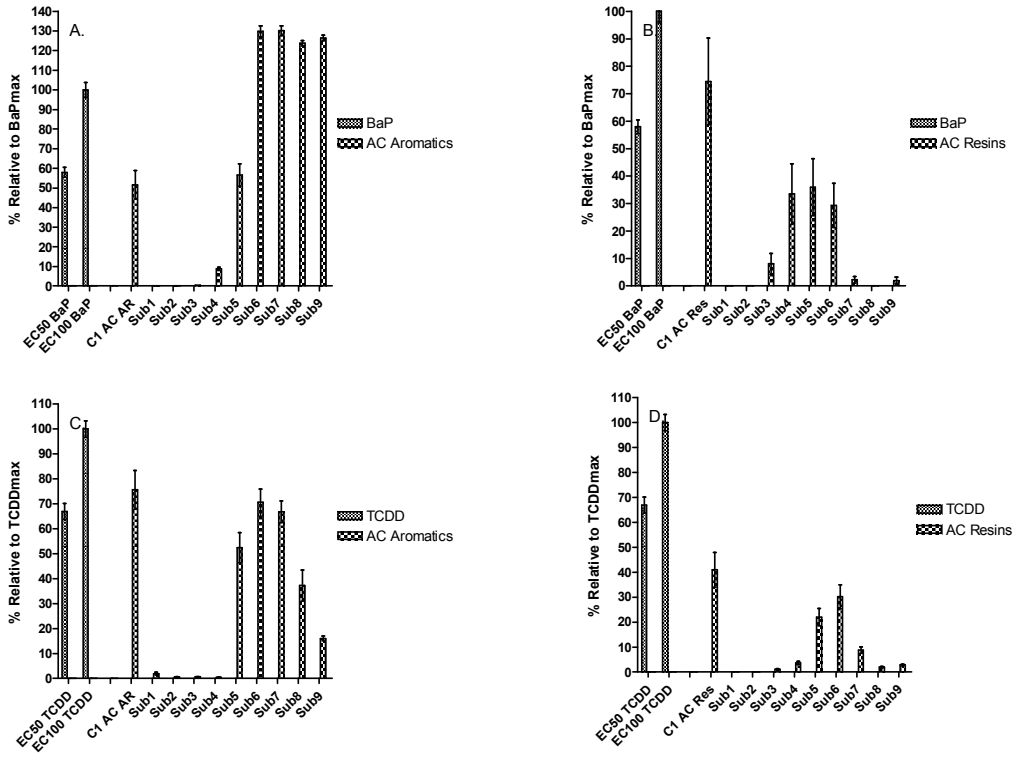

Figure 5.3. Responses to the aromatic and resin fractions of Arabian Crude (AC) oil, as well as their nine sub-fractions after 6 h (A and B) and 24 h (C and D) of exposure in the DR-Luc assay.

Translating the sub-fraction numbers to logK_{ow} ranges indicates that most of the aromatic AhR agonists are very hydrophobic compounds with logK_{ow} values between 6 and 8. Also, some less hydrophobic agonists are present in most oils (logK_{ow} 5-6), as well as some extremely hydrophobic ones (particularly in the bunker oils), with logK_{ow} >9. Interestingly, compounds with logK_{ow} >9 apparently can be transported over the cell membranes and bind

to the AhR. Assuming the absence of a hydrophobicity cut-off for bioaccumulation [37,38] and bioaccumulation factors or membrane lipid-water partition coefficients to be equal to or higher than logK_{ow} values (for 4 to 6 ring PAHs both bioaccumulation factors and membrane lipid-water partition coefficients can be up to one log unit higher than K_{ow}s; [37,38]), it is obvious that the bioaccumulation potential of the petrogenic aromatic AhR agonists is very to extremely high. For the resins, the estimated logK_{ow} range of the main agonists is somewhat lower, i.e. 5-7; however also this group contains some extremely hydrophobic, thus extremely bioaccumulative agonists (logK_{ow}s 8-10) in the bunker oils, as well as some more hydrophilic ones (logK_{ow} 3-5). According to international criteria (e.g., [39]), the majority of the AhR agonists in the oils tested should therefore be classified as 'very bioaccumulative' (vB). Additionally considering (1) the chronic toxicity of AhR agonists (PAH- and/or dioxin-like) and (2) that the agonists are most probably (relatively) persistent, as observed during the bioaccumulation experiment, as suggested by the resistance to biotransformation observed for many agonists, and also as suggested from field experiments in which P450 induction was observed for aged oils [40]; they most probably are potential PBT candidates. Obviously, this classification is not officially possible, as the identity of the chemicals is still unknown and other, officially-approved tests are required for any PBT classification.

Mass balance calculations for the HPLC fractionations (summing the 24 h TEQ values of all sub-fractions and comparing the results to the activity of the unfractionated aromatic or resin fractions) yielded recoveries for the aromatics being all > 100% (i.e., 113 to 741 %) and recoveries for the resins being close to 100% for all oils, except for the B180 and B500 bunker oils, for which recoveries measured 474 and 12 %, respectively (Table A2). As discussed above, recoveries > 100% for the aromatics of all oils and the B180 resins indicate antagonistic effects in the main fractions (before HPLC fractionation) samples. Although uncertainties in the interpolations may explain small deviations from 100%, the extremely high recoveries observed here (up to 741%) provide solid evidence for such mixture effects. The very low recovery for the B500 resins can be explained by the fact that only for this oil not all compounds were eluted from the HPLC column (the UV and fluorescence signals that were recorded during the fractionations (results not shown) were still high for this oil after collecting the last sub-fraction). Interestingly, this would imply the presence of even more hydrophobic and bioaccumulative resin agonists (i.e., logK_{ow} > 10). Theoretically, however, the occurrence of synergistic effects in the main resin fraction could also explain the low recovery value.

Preliminary chemical characterization of active sub-fractions

It is extremely challenging, if not impossible to identify the individual AhR agonists in the tested oils. The oils probably contain dozens of active compounds, which are present in a complex mixture of many thousands of hydrocarbons. Therefore, it was beyond the scope of the present study to try to attribute the *in vitro* responses to specific petrochemicals. Still,

the present experiments provide the opportunity to at least characterize the active fractions to some extent. The SARA fractionations first of all excluded saturates and asphaltenic compounds (i.e., straight-chain and branched aliphatic compounds and very high-molecular-weight complex structures, respectively) as AhR agonists. Instead, the aromatic and resin-like chemicals, such as alkylbenzenes, (alkylated) polycyclic aromatics, or aromatic chemicals containing nitrogen, sulphur, or oxygen atoms (including heterocyclic compounds) seem to be the AhR agonists. The hydrophobicity profiling does not provide much additional information on the chemical nature of the agonists, as the logK_{ow} of for instance an aromatic compound does not reveal the number of rings (alkylation also increases hydrophobicity). The same applies to the GC-FID analyses, which basically give information on the boiling point ranges of the active sub-fractions. However, these analyses did indicate that the active sub-fractions generally had high boiling points and were present mainly in the (linear alkane) range C_{14} - C_{32} (Figures A7-A8). Acute toxicity (narcosis) of fresh oils has been attributed to the low boiling point fractions (<C_{19}) [41], while our GC-FID results and those from the hydrophobicity profiling demonstrate that AhR-mediated (chronic) effects are to be expected mostly from the fractions that will not evaporate or dissolve substantially. These fractions will have a higher environmental persistence and by way of sorption to sediments or soils, their toxic potential may be preserved for prolonged times [28].

Based on the GC-MS analyses, a number of individual compounds were identified in the active fractions; mostly in the aromatic sub-fractions and only few in the resin sub-fractions (Tables A3-A4). The aromatic compounds included several alkylbenzenes, (alkylated) PAHs, alkylated biphenyls, and (alkylated) (di)benzothiophenes, whereas the resin compounds included substituted pyridines and dibenzofurans, and (alkylated/benzo-) carbazoles. We did not perform confirmation experiments with these chemicals, but most of them are known to be AhR inducers [18,31,42]. Still, probably many more chemicals will have contributed to the overall activity of the oils, as for instance no chemicals were identified in sub-fractions 8 and 9 of the oils (except for some alkylbenzenes), even though these sub-fractions did cause significant responses. Also, considering the high DR-Luc responses of the resins, the low number of resins identified, as well as the fact that carbazole (one of the few identified resins) has a relatively low AhR potency [18], only a minor fraction of the total number of resin agonists will have been identified. Identifying individual resins was difficult, because of the substantial background, which is due to the relatively high complexity of the resin sub-fractions. Additional HPLC fractionations (e.g., based on polarity) and/or more sophisticated analyses, such as GCxGC-ToF-MS would be needed to characterize the sub-fractions in more detail. As the toxicity of dioxin-like compounds (teratogenic) is totally different that of PAH-like compounds (inducing DNA adducts), additional toxicity evaluations should be performed to determine more specifically the mechanisms of toxicity of the compounds. This could be done e.g. with prolonged early life stage tests with fish larvae [43] or with the in vitro UMU assay for DNA damage [24].

The chemicals identified in separate sub-fractions were finally used to check the performance of the HPLC hydrophobicity profiling approach. LogK_{ow} values of all identified

compounds were modeled with SPARC software (Tables A3-A4) and it was tested if the resulting values corresponded with the logK_{ow} range of the sub-fraction in which the chemicals were identified. In most of the cases, chemicals indeed fell within the expected range (Tables A3-A4), thereby supporting the validity of the approach. Outliers most probably can be explained by uncertainties in predicted logK_{ow} values and/or the identification of the chemicals (e.g. substitution patterns cannot be resolved by GC-MS, but do influence hydrophobicity). All in all, the method can be considered a useful approach for profiling the hydrophobicity and bioaccumulation potential of complex mixtures, including e.g. sediment and soil extracts. Its value primarily lies in its remarkable linear behavior over a logK_{ow} range of at least eight orders of magnitude.

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Appendix Chapter 5

Detailed description of oil fractionations

1. SARA fractionation

Deasphaltenation

Approximately 0.1 g of each oil was weighed into a 10 ml vial, dissolved in 4 ml of n-hexane, and the obtained oil solutions were shaken overnight in upright position, at 20 °C, on a rotation shaker at 250 rpm. The next day, the vials were centrifuged for 7 minutes at 1500 rpm and the resulting supernatant (about 3 ml) was collected in a 100 ml brown-colored pointed flask. Next, approximately 3 ml of n-hexane was added to the vials containing the precipitated asphaltenes and the vials were shaken again at 20 °C and 250 rpm on the shaker for approximately 1 h in the dark. The washing steps were repeated until the supernatant obtained after centrifugation was colorless. The number of washing steps ranged from 6 for DMA up to 35 for B500. After each washing step, the supernatant (i.e. hexane containing deasphaltenated oil) was collected in the pointed flasks. After the last washing step, the remaining hexane in the vials was evaporated under nitrogen. The resulting precipitated, dry asphaltenes were then dissolved in 1 ml of toluene and stored at 4 °C until further use. The extracts in the pointed flasks were concentrated to 1.5 ml, using a modified Kuderna-Danish setup and nitrogen, respectively.

Separation of the saturates, aromatics, and resins

The separation of saturates, aromatics, and resins was based on EPA Method 3611 B [1]. First, glass columns were packed with glass wool, 9 g of aluminum oxide (90 active neutral) and 1 cm (approximately 125 mg) of anhydrous sodium sulfate (Na_2SO_4), respectively. The aluminum oxide had been preconditioned overnight at 130 °C and the sodium sulfate had been dried for 3 h at 600 °C. Prior to use, the columns were first pre-eluted with 50 ml of n-hexane. Then, the 1.5 ml of deasphaltated oil in hexane was transferred onto the column, which was subsequently eluted with an additional 13.5 ml of n-hexane. The eluates were collected in 100 ml pointed flasks. Next, the aromatic fractions and resins fractions were eluted by using 100 ml of dichloromethane and 100 ml of methanol, respectively. The aromatic and resin fractions were collected separately in 250 ml brown-colored pointed flasks. All resulting fractions were concentrated to 1 ml by using the modified Kurderna-Danish setup and nitrogen, respectively. The solvents in the flasks were then exchanged to toluene or ethanol as follows: to the 1 ml of saturate fractions in hexane, 2.5 ml of toluene was added; to the 1 ml of aromatic fractions in dichloromethane, 3 ml of toluene was added; and to the 1 ml of resin fractions in methanol, 5 ml of ethanol was added. All the solvent mixtures were then evaporated to exactly 1 ml under nitrogen. Full exchange to the target solvents had been confirmed by refraction index measurements in a pilot evaporation experiment.

The column material was dried in a fume hood and then extracted (shaken for 30 min on a rotation shaker) three times with 10 ml of toluene. The extracts were pooled and concentrated to exactly 1 ml as described above.

All the fractions, including the asphaltenes and the column extracts were finally diluted 20 times in ethanol.

For quality control, a procedural blank was included, which consisted of an oil-free sample and served to detect any contamination resulting from the analytical procedure.

2. HPLC fractionation

Background

The octanol-water partition coefficient (K_{ow}) is a measure of the hydrophobicity of a substance and one of the key parameters required to describe the biological activity of an organic chemical. Reversed-phase high-performance liquid chromatography (RP-HPLC) has been described many times as a technique for the indirect estimation of log K_{ow} [2-5]. The reversed-phase chromatographic retention *k*' correlates (officially a quadratic relationship, but practically a linear one) with log K_{ow} in an isocratic run. The chromatographic property used most to correlate with log K_{ow} is the retention factor for water as the mobile phase (k_w), which is usually obtained by extrapolation from isocratic measurements of *k*' for several binary solvent compositions. This works reasonably well within a homogeneous group of chemicals, although with structurally-unrelated analytes specific interactions with for example active silanol groups may play a role.

This makes RP-HPLC also a suitable technique for separating a mixture in different fractions according to hydrophobicity. Problem with this method is however the very long retention times during isocratic elution for the more hydrophobic substances. This problem can be solved by using gradient elution, instead of isocratic elution.

Different researchers have investigated the relation of retention during RP-HPLC in linear gradient runs with K_{ow}. No unambiguous compound-independent simple relationship between retention and log K_{ow} has been found however [4,6-10]. Therefore, in this study we chose an empirical approach, in which reference compounds (with known log K_{ow} values) are used to bracket the different fractions.

Method development

The aromatic and resin fractions of eight different oils were further fractionated in fractions with different log K_{ow} values by transferring the samples to a C_{18} reversed phase HPLC column and applying a gradient elution. This way, the most polar compounds will elute first and the most hydrophobic compounds will elute last; in theory (if only dispersive forces are responsible for the retention), there should be a linear relation between retention time and log K_{ow} of the compounds. The elution pattern was however calibrated with 37 different

compounds, known to occur in oils and having a known log K_{ow} value, such that a translation of retention time intervals to log K_{ow} intervals could be made. The gradient elution parameters were optimized to result in a linear dependence between log K_{ow} and the elution (retention) time.

The calibration compounds included four furans, five pyrroles, seven thiophenes, five pyridines, and sixteen PAHs, with log K_{ow} values covering a broad log K_{ow} range (*ca.* 0.5 – 8). The 37 different compounds were mixed into three standard mixes (I, II and III, all in 100% methanol). These mixes were diluted to 50/50 water/methanol before injection, in order to improve the peak shapes, as the eluting program started with 50/50 water/methanol. All compounds in one mix could be separated (baseline resolved) with the elution program used; so only three injections were needed to calibrate the elution process. Later on, the procedure was changed and the three standard mixes were diluted by a factor of 20 or more in ethanol. This was done, as the oil fractions were also dissolved in 100 % ethanol (they could not be dissolved in 50/50 water/methanol due to solubility problems). This change caused peak distortion for especially the early eluting compounds, but the retention time did not change significantly. During the eluting process, UV signals at two wavelengths (254 and 215 nm) and fluorescence (255/405 nm) signals were recorded. During the elution of the oil fractions, 8 sub-fractions were collected, which consisted of circa 12 ml of water/methanol mixture.

Analytical equipment

The HPLC system consisted of a Varian 9012 solvent delivery system, a Jasco FP-920 fluorescence detector, a Shimadzu SPD 10AVvp UV-vis detector, and a manually-operated Rheodyne 7125 analytical injector with a 100 µl sample loop. The column was an Alltima C_{18}, 150 x 7 mm (5 µm particle size) column with a Phenomenex C_{18} 4.0 x 3.0 mm guard column.

Sample preparation

All 16 original sample fractions (8 aromatic fractions and 8 resin fractions) were in 1.0 ml of 100% ethanol. The resin fractions already were dissolved in this solvent, but the aromatic fractions had been exchanged to ethanol from toluene (0.5 ml of toluene + 8 ml of pure ethanol was concentrated to 1.0 ml). Before injection, all samples were put in an ultrasonic bath for 30 minutes and were left at room temperature overnight to allow any non-dissolved precipitates to settle (to prevent clogging of the tubing/column in the HPLC-system).

Sample sub-fractionating

Resin fractions

One hundred µl of standard mixes containing the above-mentioned calibration compounds were injected in the HPLC. The HPLC gradient program used was as follows: from 0 to 45 minutes a linear gradient from 50/50 water/methanol to 0/100 water/methanol; from 45 to 80 minutes the composition was held stable at 0/100 water/methanol; from 80 to 90 minutes the system was equilibrated at 50/50 water/methanol again. The flow was 1.5 ml/min. Next, 100 µl of oil fraction samples were injected in the HPLC and the eluates were collected in 20 ml scintillation vials, covered with aluminum foil and containing 3 ml of n-hexane. The retention time intervals of the collected eluate samples corresponded to log K_{ow} intervals of 0-2; 2-3; 3-4; 4-5; 5-6; 6-7; 7-8; 8-9 and 9-10.5, as calculated using linear regression (retention time vs. log K_{ow}). Actual times and logK_{ow} intervals are presented in the table below.

Fraction	Starting time sampling (min)	Ending time sampling (min)	log K_{ow} start	log K_{ow} end
1	2:10	13:45	0.00	2.03
2	13:45	21:33	2.03	3.02
3	21:33	29:23	3.02	4.01
4	29:23	37:10	4.01	5.00
5	37:10	45:00	5.00	5.99
6	45:00	52:49	5.99	6.98
7	52:49	60:38	6.98	7.98
8	60:38	68:26	7.98	8.97
9	68:26	80:00	8.97	10.43

In order to check whether the correlation between retention times and logK_{ow} remained constant, the three standard mixes were injected in between the sample injections.

After elution/fractionation of each sample, a new guard column was installed to prevent retained or very slow eluting compounds from interfering with the next fractionation. The collected resins sub-fractions were stored at 4 °C, until further use.

Aromatic fractions

The procedure used to fractionate the aromatics was similar to the one used for the resins, with the following addition. After elution/fractionation of each sample, the column including the guard column was reversely flushed for 80 minutes with 100% methanol. The

signal of this back-flushing was recorded. Before the next injection, 10 minutes of equilibration at 50/50 water/methanol was performed. The collected aromatic sub-fractions were stored at 4 °C until further use. Retention time intervals again corresponding to log K_{ow} intervals of 0-2; 2-3; 3-4; 4-5; 5-6; 6-7; 7-8; 8-9 and 9-10.5 were calculated using linear regression. Actual times and log K_{ow} intervals are presented in the table below.

Fraction	Starting time sampling (min)	Ending time sampling (min)	log K_{ow} start	log K_{ow} end
1	2:10	13:34	0.00	1.98
2	13:34	21:18	1.98	2.98
3	21:18	29:02	2.98	3.99
4	29:02	36:46	3.99	4.99
5	36:46	44:30	4.99	6.00
6	44:30	52:14	6.00	7.00
7	52:14	59:58	7.00	8.01
8	59:58	67:42	8.01	9.01
9	67:42	80:00	9.01	10.61

Extractions of the methanol-water eluates and solvent exchange

In order to be able to dose the sub-fractions in the in vitro assay, it was necessary to reduce the volume to ca. 1 ml and to change the solvent to ethanol. This was done as follows. Samples (± 12 ml + 3 ml of hexane) were quantitatively transferred to a volumetric cylinder of 100 ml with stopper and about 200 mg of $CaCl_2$ was added. Next, 17 ml of n-hexane was added (via the 20 ml vial, which was as such rinsed 3 times) and the cylinder was filled up to 100 ml with Millipore water. The cylinder was shaken vigorously for 3 minutes and the hexane layer was transferred to a 100 ml pointed flask. The addition, shaking, and transferring of n-hexane was then repeated twice. The pooled hexane extract (*ca.* 60 ml) was subsequently concentrated to 0.5 ml, using a modified Kuderna-Danish setup and nitrogen, respectively. Next, 1.5 ml of ethanol was added and the extract was concentrated again with nitrogen to ≤ 0.5 ml. Ethanol was finally added to a volume of 1.0 ml exactly.

Two types of blank samples were included in this procedure; one involving the extraction of 12 ml of methanol and one involving the extraction of 12 ml of water/methanol 50/50. All final extracts in ethanol were stored in the freezer.

The resin sub-fractions of the B500 oil were additionally extracted a second time after the first extractions with hexane, in order to check whether acidic toxic chemicals (e.g. naphtenic acids) might not have been extracted to the hexane layer. To this end, the content of the cylinders (containing the diluted water/methanol eluate) was acidified with 1 ml of 1 M HCl and extracted an additional three times with hexane. The resulting extracts did

however not show any responses in the DR-Luc assay and therefore are not included in Figures A5 and A6.

Recovery check of the extraction and solvent exchange procedure

The above extraction procedure (phase ratio and extraction time) was designed based on a recovery test with a selection of compounds. The following compounds were used: furan, 2-methylfuran, dibenzofuran, pyrrole, benzo(b)pyrrole, 9-ethylcarbazole, benzothiophene, pyridine, benzo[b]quinoline, benzofuran, 3-methylthiophene, 2-propylthiophene, 3-octylthiophene, benzo[b]naphthol[2,1-d]thiophene and 4-methylquinoline. The experiment involved different phase ratios and extraction times, based on the results of which the above protocol was selected for its highest yields. A recovery experiment with all PAHs had been carried out previously and indicated that the above-described procedure resulted in recoveries > 98%.

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Detailed description of the in vitro assays

Dioxin receptor-mediated luciferase reporter gene (DR-Luc) assay and chemically activated fluorescence assay (CAFLUX).

In order to identify the fractions and sub-fractions of oil responsible for activating the AhR, the dioxin receptor-mediated luciferase reporter gene assay (DR-Luc) was used. The extracts of oil-contaminated sediments and worms exposed to these sediments were investigated by employing the chemically activated fluorescence assay (CAFLUX). Both assays are based on the principle that specific chemicals can activate the AhR, resulting in a cascade of molecular events leading to a dose-dependent expression of the luciferase (DR-Luc assay) or fluorescence protein (CAFLUX assay). In the DR-Luc assay AhR-activation also results in induction of cytochrome P450 1A, which can metabolize planar aromatic compounds, including polycyclic aromatic hydrocarbons (PAHs). In the CAFLUX assay the produced reporter protein is stable, so this assay gives a measure of the total AhR-agonistic activity. In the DR-Luc assay the luciferase protein degrades after a few hours, allowing the distinction between stable AhR agonists (such as TCDD) that still are present after 24 or 48 hours, and easily metabolizable compounds (such as BaP) that will be disappeared within a few hours [1].

Cells culturing conditions

The DR-Luc cells are a rat hepatoma cell line (H4IIE.luc), stably expressing the luciferase gene, and were kindly provided by Prof. dr. M. Denison (UCD, USA). The cells were cultured in 75 cm^2 culture flasks (Corning), in 10 ml minimal essential medium without geneticine (α-MEM; Gibco-Invitrogen), but supplemented with 10% heat-inactivated fetal calf serum (FCS), at 37 °C and 5% CO_2.

The CAFLUX cells are a mouse hepatoma cell line (H1G1), stably expressing the fluorescent AHR construct, and were also kindly provided by Prof. dr. M. Denison. The cells were cultured in 75 cm^2 culture flasks (Cellstar®, Greiner) in 14 ml DMEM/F12 (Gibco), supplemented with 10 % FCS and 1 % penicillin and streptomycin (Gibco), at 37 °C and 5% CO_2.

Exposure of the cells to (sub-)fractions of oils in the DR-Luc assay

The cells were exposed for 6 or 24 h to the (sub-)fractions of oils according to a method previously described [2,3]. One day before the exposure, a 70-90 % confluent layer of cells was trypsinized, diluted, and subsequently pipetted into each of the 60 center wells of 96-well plates (Packard, ViewPlate™-96, white, PerkinElmer Life and Analytical Sciences, Groningen, The Netherlands). The volume used was 100 µl cell suspension per well. The plates were incubated overnight to allow the cells to reach 100% confluency. On the

exposure day, another 100 µl of medium was added in each well. Next, the cells were exposed to 1 µl of (sub-)fraction of the different oils, such as that the concentration of the oil fraction in case of the SARA fractions was 25 mg oil/ L medium, corresponding to a previously-tested concentration of whole oils [3]. On each plate the following controls were included: benzo[*a*]pyrene (BaP) (12.5 pM to 125 nM) for 6 exposures or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (0.125 to 375 pM) for 24 h exposures, respectively; a medium control; and a 0.5% ethanol control. All the concentrations were tested in triplicate on a single plate and the experiments were performed twice.

Exposure of cells to worm and sediment extracts in the CAFLUX assay

The 90% confluent H1G1.c3 cells were washed, trypsinized, and diluted to 2×10^5 cells/ml in DMEM/F12 medium. The homogenized, diluted cell suspension was divided over sterile glass tubes. This way of dosing the cells was preferred in this case, as pilot experiments had demonstrated that better dose-response curves were obtained for the standards. Next, the cells were exposed to the 200 times diluted worm and sediment extracts. As a positive control, a 1.35 pM to 9 nM concentration range of TCDD in ethanol was used. Medium and a 0.5 % ethanol solution were used as negative controls. After a gentle homogenization, the medium containing the cells and extracts were transferred to the inner 60 wells of a 96-wells plate. Each concentration was tested in triplicate and the exposure of cells to the extracts lasted for 24 h.

Response quantification in the DR-Luc assay

Response quantification in the luciferase assays was performed as previously described [3,4]. To summarize, at the end of the exposure time, the medium was removed, the cells were washed with 0.5 x PBS, and lysed with low-salt hypotonic buffer. In order to allow swelling of the cells, the plates were placed on ice for 10 min and then frozen at -80 ^{o}C for at least 30 min, until use. Just before measurement, the plates were thawed at room temperature for 1 hour and then shaken on a plate shaker for 2 min. Next, the luciferase production was measured in a luminometer (Labsystems, Luminoskan RS, Helsinki, Finland).

The responses of the (sub)fractions of oils were expressed as percentage responses, relative to the maximum response of the assays' standard (BaPmax or TCDD max), after the subtraction of the background response of the solvent control (ethanol).

Response quantification in the CAFLUX assay

At the end of exposure time, the medium was removed and the cells were washed with 200 µl of PBS. The PBS was removed and the wells were filled again with 200 µl of fresh PBS. The fluorescence was then measured on a FLUOstar (BMG, Labtechnologies), at an excitation wavelength of 485 nm and an emission wavelength of 510 nm.

References

[1] Hamers T, van Schaardenburg MD, Felzel EC, Murk AJ, Koeman JH. 2000. The application of reporter gene assays for the determination of the toxic potency of diffuse air pollution. *Sci Total Environ* 262:159-174.

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[3] Vrabie CM, Jonker MTO, Murk AJ. 2009. Specific in vitro toxicity of crude and refined petroleum products. 1. Aryl hydrocarbon receptor-mediated responses. *Environ Toxicol Chem* 28:1995-2003.

[4] ter Veld MGR, Schouten B, Louisse J, van Es DS, van der Saag PT, Rietjens IMCM, Murk AJ. 2006. Estrogenic potency of food-packaging-associated plasticizers and antioxidants as detected in ERα and ERβ reporter gene cell lines. *J Agric Food Chem* 54:4407-4416.

Table A1. Abbreviations, applications, sources, and/or suppliers of the eight oils tested.

Name	Abbreviation	Source/supplier	Application
Refined products			
Distillate marine grade A	DMA	Gulf Oil, Nigtevecht, The Netherlands	Gasoil in midsized to larger ships, such as coasters, large cutters, and inland vessels
Bilge Oil	BIL	Oil layer scooped from a bilge water collection depot at a yacht-basin, Wageningen	None; waste product
Bunker 180 (Heavy nautical fuel oil)	B180	Atlantic BV oil company, Pernis, The Netherlands	Fuel in large ships
Bunker 500 (Heavy nautical fuel oil)	B500	Atlantic BV oil company, Pernis, The Netherlands	Fuel in large ships
Crude oils			
Arabian crude	AC	Shell refinery Pernis, The Netherlands; extracted in Saudi Arabia	Used for refining into petroleum products
Romanian crude	RC	Extracted at the Tintea oil field, Ploiesti, Romania	Used for refining into petroleum products
Oseberg crude	OC	Shell refinery Pernis, The Netherlands; extracted at the Oseberg area, Norway	Used for refining into petroleum products
Hollmix crude	HMC	Shell refinery Pernis, The Netherlands; offshore product from the North Sea	Used for refining into petroleum products

Table A2. Recovery % of the SARA and HPLC fractionations, as calculated by summing the activities (mg TEQ) in the fractions and comparing to the initial activity of the unfractionated sample.

Oil	% recovery after SARA fractionation	% recovery after HPLC fractionation aromatics	% recovery after HPLC fractionation resins
DMA	44	167	88
BIL	477	134	134
B180	35	555	474
B500	74	741	12
AC	56	278	79
RC	46	175	100
OC	39	172	97
HMC	35	113	120

Table A3. Presence of selected aromatic compounds in active aromatic sub-fractions, as identified by GC-MS analyses and indicated by bullets. See below the last table for an explanation of fraction codes.

	search mass	MW	DA2	DA3	DA4	DA5	DA6	DA7	DA8	DA9	SPARC log K_{ow}
n-Alkylbenzenes											
C1-benzene (methyl)	91+92	92									
C2-benzene (ethyl)	91+106	106									
C3-benzene (trimethyl)	91+120	120			•						3.7
C4-benzene (tetramethyl)	91+92+134	134									4.11
C5-benzene (pentamethyl)	91+92+148	148				•					4.57
C6-benzene (hexamethyl)	91+92+162	162				•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176				•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190					•				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204					•				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218					•				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232					•				7.23
C12-benzene (hexaethyl-)	91+92+246	246					•	•			7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260						•			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274						•			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288						•			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302							•		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316							•		10.15
C18-benzene (hexapropyl-)	91+92+330	330								•	10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344								•	11.12
Benzene, propenyl / Indane	117+118	118		•							3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132			•						4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146			•	•					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148			•	•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160				•					4.64 / error
Naphthalene/Azulene/methylene Indene	128	128		••							3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130		•							3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142			••						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156			•						4.5 / 4.48
Naphthalene, trimethyl	155+170	170				••					4.99
Naphthalene, tetramethyl	169+184	184				•					error
Naphthalene, tetrahydro-methyl	131+146	146				•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160				•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174				•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170				•					5.04
Naphthalene, dimethyl, phenyl	202+217+232	232					•				error

	search mass	MW	DA2	DA3	DA4	DA5	DA6	DA7	DA8	DA9	SPARC log K_{ow}
Fluorene	165+166	166			●●						4.36
Fluorene, methyl	165+180	180			●	●					4.85
Fluorene, dimethyl	179+194	194				●					5.28
Benzofluorene / Pyrene, methyl	215+216	216				●					5.81 /5.84
Phenanthrene		178			●●						4.9
Phenanthrene, methyl	191+192	192				●●					5.34
Phenanthrene, dimethyl	191+206	206				●					5.64
Phenanthrene, trimethyl	205+220	220				●	●				6.03
Phenanthrene, tetramethyl	219+234	234					●				6.48
Pyrene/Fluoranthene		202				●					5.39/5.46
Perene, dimethyl	215+229+230	230									6.3
B[a]A/chrysene	228	228									6.01/6.06
B[a]A, methyl	242	242									6.37
B[k]F/B[a]P	126+252	252									6.68/6.7
B[k]F, methyl		266									error
B[k]F, dimethyl		280									error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234									6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248									6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262									7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276									
Benzo[b]thiophene,	134	134		●							3.21
Benzo[b]thiophene, methyl	147+148	148		●							3.74
Benzo[b]thiophene, dimethyl	161+162	162			●						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176				●					4.84
Dibenzothiophene		184			●●						4.52
Dibenzothiophene, methyl	197+198	198				●					5.04
Dibenzothiophene, dimethyl	211+212	212				●					5.47
Dibenzothiophene, trimethyl		226					●				6.03
Biphenyl	153+154	154			●						4.31
Biphenyl, methyl	153+167+168	168			●●						4.78
Biphenyl, dimethyl	167+182	182				●					5.25
Biphenyl, trimethyl		196					●				5.74
Benzylquinoline	218	219									4.35

	search mass	MW	BA5	BA6	BA7	BA8	BA9	SPARC log K_{ow}
n-Alkylbenzenes								
C1-benzene (methyl)	91+92	92						
C2-benzene (ethyl)	91+106	106						
C3-benzene (trimethyl)	91+120	120						3.7
C4-benzene (tetramethyl)	91+92+134	134						4.11
C5-benzene (pentamethyl)	91+92+148	148						4.57
C6-benzene (hexamethyl)	91+92+162	162	•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176	•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190		•				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204		•				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218		•				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232		•				7.23
C12-benzene (hexaethyl-)	91+92+246	246		•	•			7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260			•			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274			•			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288			•			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302				•		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316				•		10.15
C18-benzene (hexapropyl-)	91+92+330	330					•	10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344					•	11.12
Benzene, propenyl / Indane	117+118	118						3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132						4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146	•					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148	•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.64 / error
Naphthalene/Azulene/methylene Indene	128	128						3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130						3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156						4.5 / 4.48
Naphthalene, trimethyl	155+170	170	••					4.99
Naphthalene, tetramethyl	169+184	184	•					error
Naphthalene, tetrahydro-methyl	131+146	146	•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174	•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170						5.04
Naphthalene, dimethyl, phenyl	202+217+232	232		•				error

	search mass	MW	BA5	BA6	BA7	BA8	BA9	SPARC log K_{ow}
Fluorene	165+166	166						4.36
Fluorene, methyl	165+180	180	•					4.85
Fluorene, dimethyl	179+194	194	•					5.28
Benzofluorene / Pyrene, methyl	215+216	216	•					5.81 /5.84
Phenanthrene		178						4.9
Phenanthrene, methyl	191+192	192	••					5.34
Phenanthrene, dimethyl	191+206	206	•					5.64
Phenanthrene, trimethyl	205+220	220	•	•				6.03
Phenanthrene, tetramethyl	219+234	234		•				6.48
Pyrene/Fluoranthene		202	•					5.39/5.46
Perene, dimethyl	215+229+230	230						6.3
B[a]A/chrysene	228	228						6.01/6.06
B[a]A, methyl	242	242						6.37
B[k]F/B[a]P	126+252	252						6.68/6.7
B[k]F, methyl		266						error
B[k]F, dimethyl		280						error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234						6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248						6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262						7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276						
Benzo[b]thiophene,	134	134						3.21
Benzo[b]thiophene, methyl	147+148	148						3.74
Benzo[b]thiophene, dimethyl	161+162	162						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176						4.84
Dibenzothiophene		184						4.52
Dibenzothiophene, methyl	197+198	198	•					5.04
Dibenzothiophene, dimethyl	211+212	212	•					5.47
Dibenzothiophene, trimethyl		226	•	•				6.03
Biphenyl	153+154	154						4.31
Biphenyl, methyl	153+167+168	168						4.78
Biphenyl, dimethyl	167+182	182	•					5.25
Biphenyl, trimethyl		196	•	•				5.74
Benzylquinoline	218	219						4.35

	search mass	MW	180A3	180A4	180A5	180A6	180A7	180A8	180A9	SPARC log K_{ow}
n-Alkylbenzenes										
C1-benzene (methyl)	91+92	92								
C2-benzene (ethyl)	91+106	106								
C3-benzene (trimethyl)	91+120	120								3.7
C4-benzene (tetramethyl)	91+92+134	134								4.11
C5-benzene (pentamethyl)	91+92+148	148								4.57
C6-benzene (hexamethyl)	91+92+162	162			•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176			•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190				•				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204				•				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218				•				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232								7.23
C12-benzene (hexaethyl-)	91+92+246	246					•			7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260					•			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274					•			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288					•			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302						•		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316						•		10.15
C18-benzene (hexapropyl-)	91+92+330	330							•	10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344							•	11.12
Benzene, propenyl / Indane	117+118	118	•							3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132		•						4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146		•	•					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148			•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160			•					4.64 / error
Naphthalene/Azulene/methylene Indene	128	128	••							3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130	•							3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142		••						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156		••						4.5 / 4.48
Naphthalene, trimethyl	155+170	170			••					4.99
Naphthalene, tetramethyl	169+184	184			•					error
Naphthalene, tetrahydro-methyl	131+146	146			•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160			•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174			•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170			•					5.04
Naphthalene, dimethyl, phenyl	202+217+232	232				•				error

	search mass	MW	180A3	180A4	180A5	180A6	180A7	180A8	180A9	SPARC log K_{ow}
Fluorene	165+166	166		•						4.36
Fluorene, methyl	165+180	180		•	•					4.85
Fluorene, dimethyl	179+194	194			•					5.28
Benzofluorene / Pyrene, methyl	215+216	216			•					5.81 /5.84
Phenanthrene		178		••						4.9
Phenanthrene, methyl	191+192	192			•					5.34
Phenanthrene, dimethyl	191+206	206			•					5.64
Phenanthrene, trimethyl	205+220	220			•	•				6.03
Phenanthrene, tetramethyl	219+234	234				•				6.48
Pyrene/Fluoranthene		202			•					5.39/5.46
Perene, dimethyl	215+229+230	230				•				6.3
B[a]A/chrysene	228	228			•					6.01/6.06
B[a]A, methyl	242	242			•	•				6.37
B[k]F/B[a]P	126+252	252				•				6.68/6.7
B[k]F, methyl		266				•				error
B[k]F, dimethyl		280					•			error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234			•					6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248				•				6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262				•				7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276				•	•			
Benzo[b]thiophene,	134	134								3.21
Benzo[b]thiophene, methyl	147+148	148								3.74
Benzo[b]thiophene, dimethyl	161+162	162		•						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176			•					4.84
Dibenzothiophene		184		••						4.52
Dibenzothiophene, methyl	197+198	198			•					5.04
Dibenzothiophene, dimethyl	211+212	212			•					5.47
Dibenzothiophene, trimethyl		226				•				6.03
Biphenyl	153+154	154		••						4.31
Biphenyl, methyl	153+167+168	168		••	•					4.78
Biphenyl, dimethyl	167+182	182			•					5.25
Biphenyl, trimethyl		196								5.74
Benzylquinoline	218	219			•					4.35

	search mass	MW	500A3	500A4	500A5	500A6	500A7	500A8	500A9	SPARC log K_{ow}
n-Alkylbenzenes	**mass**									
C1-benzene (methyl)	91+92	92								
C2-benzene (ethyl)	91+106	106								
C3-benzene (trimethyl)	91+120	120								3.7
C4-benzene (tetramethyl)	91+92+134	134								4.11
C5-benzene (pentamethyl)	91+92+148	148								4.57
C6-benzene (hexamethyl)	91+92+162	162			●					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176			●					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190				●				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204				●				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218				●				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232				●				7.23
C12-benzene (hexaethyl-)	91+92+246	246				●	●			7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260					●			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274					●			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288					●			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302						●		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316						●		10.15
C18-benzene (hexapropyl-)	91+92+330	330							●	10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344							●	11.12
Benzene, propenyl / Indane	117+118	118	●							3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132		●						4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146		●	●					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148			●					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160			●					4.64
Naphthalene/Azulene/methylene Indene	128	128	●●							3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130	●							3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142		●●						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156		●●						4.5 / 4.48
Naphthalene, trimethyl	155+170	170			●●					4.99
Naphthalene, tetramethyl	169+184	184			●					5.35
Naphthalene, tetrahydro-methyl	131+146	146			●					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160			●					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174 / 160			●					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170			●					5.04
Naphthalene, dimethyl, phenyl	202+217+232	232				●				6.63

	search mass	MW	500A3	500A4	500A5	500A6	500A7	500A8	500A9	SPARC log K_{ow}
Fluorene	165+166	166		●						4.36
Fluorene, methyl	165+180	180		●	●					4.85
Fluorene, dimethyl	179+194	194			●					5.28
Benzofluorene / Pyrene, methyl	215+216	216			●					5.81 /5.84
Phenanthrene	178	178		●●						4.9
Phenanthrene, methyl	191+192	192			●					5.34
Phenanthrene, dimethyl	191+206	206			●					5.64
Phenanthrene, trimethyl	205+220	220			●	●				6.03
Phenanthrene, tetramethyl	219+234	234				●				6.48
Pyrene/Fluoranthene		202			●					5.39/5.46
Perene, dimethyl	215+229+230	230				●				6.3
B[a]A/chrysene	228	228			●					6.01/6.06
B[a]A, methyl	242	242			●	●				6.37
B[k]F/B[a]P	126+252	252				●				6.68/6.7
B[k]F, methyl		266				●				7.1
B[k]F, dimethyl		280					●			7.55
Benzo[b]naphtho[2,1-d]thiophene	117+234	234			●					6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248				●				6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262				●				7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276				●	●			7.56
Benzo[b]thiophene,	134	134	●							3.21
Benzo[b]thiophene, methyl	147+148	148	●							3.74
Benzo[b]thiophene, dimethyl	161+162	162		●						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176			●					4.84
Dibenzothiophene		184		●●						4.52
Dibenzothiophene, methyl	197+198	198			●					5.04
Dibenzothiophene, dimethyl	211+212	212			●					5.47
Dibenzothiophene, trimethyl		226				●				6.03
Biphenyl	153+154	154		●						4.31
Biphenyl, methyl	153+167+168	168		●●	●					4.78
Biphenyl, dimethyl	167+182	182			●					5.25
Biphenyl, trimethyl		196				●				5.74
Benzylquinoline	218	219			●					4.35

	search mass	MW	AA4	AA5	AA6	AA7	AA8	AA9	SPARC log K_{ow}
n-Alkylbenzenes									
C1-benzene (methyl)	91+92	92							
C2-benzene (ethyl)	91+106	106							
C3-benzene (trimethyl)	91+120	120	•						3.7
C4-benzene (tetramethyl)	91+92+134	134							4.11
C5-benzene (pentamethyl)	91+92+148	148							4.57
C6-benzene (hexamethyl)	91+92+162	162		•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176		•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190			•				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204			•				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218			•				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232			•				7.23
C12-benzene (hexaethyl-)	91+92+246	246			•				7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260				•			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274				•			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288				•			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302					•		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316					•		10.15
C18-benzene (hexapropyl-)	91+92+330	330							10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344							11.12
Benzene, propenyl / Indane	117+118	118							3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132							4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146	•	•					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148	•	•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160		•					4.64 / error
Naphthalene/Azulene/methylene Indene	128	128							3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130							3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142	••						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156	••						4.5 / 4.48
Naphthalene, trimethyl	155+170	170		••					4.99
Naphthalene, tetramethyl	169+184	184		•					error
Naphthalene, tetrahydro-methyl	131+146	146		•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160		•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174		•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170							5.04
Naphthalene, dimethyl, phenyl	202+217+232	232			•				error

	search mass	MW	AA4	AA5	AA6	AA7	AA8	AA9	SPARC log K_{ow}
Fluorene	165+166	166	•						4.36
Fluorene, methyl	165+180	180		•					4.85
Fluorene, dimethyl	179+194	194		•					5.28
Benzofluorene / Pyrene, methyl	215+216	216		•					5.81 /5.84
Phenanthrene		178	•						4.9
Phenanthrene, methyl	191+192	192		•					5.34
Phenanthrene, dimethyl	191+206	206		•					5.64
Phenanthrene, trimethyl	205+220	220		•	•				6.03
Phenanthrene, tetramethyl	219+234	234							6.48
Pyrene/Fluoranthene		202							5.39/5.46
Perene, dimethyl	215+229+230	230							6.3
B[a]A/chrysene	228	228							6.01/6.06
B[a]A, methyl	242	242							6.37
B[k]F/B[a]P	126+252	252							6.68/6.7
B[k]F, methyl		266							error
B[k]F, dimethyl		280							error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234							6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248			•				6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262			•				7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276							
Benzo[b]thiophene,	134	134							3.21
Benzo[b]thiophene, methyl	147+148	148							3.74
Benzo[b]thiophene, dimethyl	161+162	162	•						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176		•					4.84
Dibenzothiophene		184	••						4.52
Dibenzothiophene, methyl	197+198	198		•					5.04
Dibenzothiophene, dimethyl	211+212	212		•					5.47
Dibenzothiophene, trimethyl		226			•				6.03
Biphenyl	153+154	154	•						4.31
Biphenyl, methyl	153+167+168	168							4.78
Biphenyl, dimethyl	167+182	182							5.25
Biphenyl, trimethyl		196							5.74
Benzylquinoline	218	219							4.35

	search mass	MW	RA5	RA6	RA7	RA8	RA9	SPARC log K_{ow}
n-Alkylbenzenes								
C1-benzene (methyl)	91+92	92						
C2-benzene (ethyl)	91+106	106						
C3-benzene (trimethyl)	91+120	120						3.7
C4-benzene (tetramethyl)	91+92+134	134						4.11
C5-benzene (pentamethyl)	91+92+148	148						4.57
C6-benzene (hexamethyl)	91+92+162	162	•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176	•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190		•				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204		•				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218		•				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232		•				7.23
C12-benzene (hexaethyl-)	91+92+246	246		•				7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260			•			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274			•			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288			•			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302				•		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316				•		10.15
C18-benzene (hexapropyl-)	91+92+330	330					•	10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344					•	11.12
Benzene, propenyl / Indane	117+118	118						3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132						4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146	•					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148	•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.64 / error
Naphthalene/Azulene/methylene Indene	128	128						3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130						3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156						4.5 / 4.48
Naphthalene, trimethyl	155+170	170	••					4.99
Naphthalene, tetramethyl	169+184	184	•					error
Naphthalene, tetrahydro-methyl	131+146	146	•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174	•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170	•					5.04
Naphthalene, dimethyl, phenyl	202+217+232	232		•				error
Biphenyl, dimethyl	167+182	182						
Biphenyl, trimethyl		196						

	search mass	MW	RA5	RA6	RA7	RA8	RA9	SPARC log K_{ow}
Fluorene	165+166	166						4.36
Fluorene, methyl	165+180	180	•					4.85
Fluorene, dimethyl	179+194	194	•					5.28
Benzofluorene / Pyrene, methyl	215+216	216	•					5.81 /5.84
Phenanthrene		178						4.9
Phenanthrene, methyl	191+192	192	••					5.34
Phenanthrene, dimethyl	191+206	206	•					5.64
Phenanthrene, trimethyl	205+220	220	•	•				6.03
Phenanthrene, tetramethyl	219+234	234		•				6.48
Pyrene/Fluoranthene		202						5.39/5.46
Perene, dimethyl	215+229+230	230						6.3
B[a]A/chrysene	228	228						6.01/6.06
B[a]A, methyl	242	242	•	•				6.37
B[k]F/B[a]P	126+252	252						6.68/6.7
B[k]F, methyl		266						error
B[k]F, dimethyl		280						error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234						6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248						6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262						7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276						
Benzo[b]thiophene,	134	134						3.21
Benzo[b]thiophene, methyl	147+148	148						3.74
Benzo[b]thiophene, dimethyl	161+162	162						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176	•					4.84
Dibenzothiophene		184						4.52
Dibenzothiophene, methyl	197+198	198	•					5.04
Dibenzothiophene, dimethyl	211+212	212	•					5.47
Dibenzothiophene, trimethyl		226		•				6.03
Biphenyl	153+154	154						4.31
Biphenyl, methyl	153+167+168	168	•					4.78
Biphenyl, dimethyl	167+182	182	•					5.25
Biphenyl, trimethyl		196		•				5.74
Benzylquinoline	218	219						4.35

	search mass	MW	OA5	OA6	OA7	OA8	OA9	SPARC log K_{ow}
n-Alkylbenzenes								
C1-benzene (methyl)	91+92	92						
C2-benzene (ethyl)	91+106	106						
C3-benzene (trimethyl)	91+120	120						3.7
C4-benzene (tetramethyl)	91+92+134	134						4.11
C5-benzene (pentamethyl)	91+92+148	148						4.57
C6-benzene (hexamethyl)	91+92+162	162	•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176	•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190		•				5.87
C9-benzene (trimethyl, triethyl-)	91+92+204	204		•				6.35
C10-benzenen (dimethyl, tetraethyl-)	91+92+218	218		•				6.79
C11-benzene (methyl, pentaethyl-)	91+92+232	232		•				7.23
C12-benzene (hexaethyl-)	91+92+246	246		•	•			7.71
C13-benzene (pentaethyl, propyl-)	91+92+260	260			•			8.21
C14-benzene (tetraethyl, dipropyl-)	91+92+274	274			•			8.7
C15-benzene (triethyl, tripropyl-)	91+92+288	288			•			9.19
C16-benzene (diethyl, tetrapropyl)	91+92+302	302				•		9.67
C17-benzene (ethyl, pentapropyl-)	91+92+316	316				•		10.15
C18-benzene (hexapropyl-)	91+92+330	330					•	10.62
C19-benzene (pentapropyl, butyl-)	91+92+344	344					•	11.12
Benzene, propenyl / Indane	117+118	118						3.58 / 3.27
Benzene, methyl, propenyl / Indene, dihydro, methyl	117+132	132						4.04 / 3.71
Benzene, ethenyl, trimethyl / Indene, dihydro, dimethyl / Naphthalene, tetrahydro-methyl	131+146	146	•					4.49 / 4.04 /4.18
Benzene, methyl, methylpropyl	119+148	148	•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.64 / error
Naphthalene/Azulene/methylene Indene	128	128						3.54 / error / 3.7
Dihydronaphthalene / Indene, methyl	115+129+130	130						3.57 / 3.62
Indene ethylidene / C1-naphthalene	115+141+142	142						4.09 / 4.02
Naphthalene, dimethyl/ Naphthalene, ethyl	115+141+156	156						4.5 / 4.48
Naphthalene, trimethyl	155+170	170	••					4.99
Naphthalene, tetramethyl	169+184	184	•					error
Naphthalene, tetrahydro-methyl	131+146	146	•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174	•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170	•					5.04
Naphthalene, dimethyl, phenyl	202+217+232	232		•				error

	search mass	MW	OA5	OA6	OA7	OA8	OA9	SPARC log K_{ow}
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Benzofluorene / Pyrene, methyl	215+216	216						5.81 /5.84
Phenanthrene		178						4.9
Phenanthrene, methyl	191+192	192	••					5.34
Phenanthrene, dimethyl	191+206	206	•					5.64
Phenanthrene, trimethyl	205+220	220	•	•				6.03
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Pyrene/Fluoranthene		202						5.39/5.46
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B[a]A/chrysene	228	228	•					6.01/6.06
B[a]A, methyl	242	242	•	•				6.37
B[k]F/B[a]P	126+252	252						6.68/6.7
B[k]F, methyl		266						error
B[k]F, dimethyl		280						error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234						6.07
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C6-benzene (hexamethyl)	91+92+162	162	•					5.00
C7-benzene (pentamethyl, ethyl-)	91+92+176	176	•					5.39
C8-benzene (tetramethyl, diethyl-)	91+92+190	190		•				5.87
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Benzene, methyl, methylpropyl	119+148	148	•					4.56
Indene, dihydro trimethyl / Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.64 / error
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Naphthalene, tetramethyl	169+184	184	•					error
Naphthalene, tetrahydro-methyl	131+146	146	•					4.18
Naphthalene-tetrahydro-dimethyl	145+160	160	•					4.52
Naphthalene, tetrahydro-trimethyl / Benzimidazole, 4,5,6,7-tetramethyl	159+174	174	•					5.11 or 5.07/ 6.02
Naphthalene, propyl	141+170	170	•					5.04
Naphthalene, dimethyl, phenyl	202+217+232	232		•				error

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Fluorene, dimethyl	179+194	194	•					5.28
Benzofluorene / Pyrene, methyl	215+216	216						5.81 /5.84
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Phenanthrene, methyl	191+192	192	•					5.34
Phenanthrene, dimethyl	191+206	206	•					5.64
Phenanthrene, trimethyl	205+220	220		•				6.03
Phenanthrene, tetramethyl	219+234	234		•				6.48
Pyrene/Fluoranthene		202						5.39/5.46
Perene, dimethyl	215+229+230	230						6.3
B[a]A/chrysene	228	228						6.01/6.06
B[a]A, methyl	242	242						6.37
B[k]F/B[a]P	126+252	252						6.68/6.7
B[k]F, methyl		266						error
B[k]F, dimethyl		280						error
Benzo[b]naphtho[2,1-d]thiophene	117+234	234						6.07
Benzo[b]naphtho[2,1-d]thiophene, methyl		248						6.58
Benzo[b]naphtho[2,1-d]thiophene, dimethyl		262						7.14
Benzo[b]naphtho[2,1-d]thiophene, trimethyl		276						
Benzo[b]thiophene,	134	134						3.21
Benzo[b]thiophene, methyl	147+148	148						3.74
Benzo[b]thiophene, dimethyl	161+162	162						4.29
Benzo[b]thiophene, trimethyl	161+175+176	176						4.84
Dibenzothiophene		184						4.52
Dibenzothiophene, methyl	197+198	198						5.04
Dibenzothiophene, dimethyl	211+212	212						5.47
Dibenzothiophene, trimethyl		226						6.03
Biphenyl	153+154	154						4.31
Biphenyl, methyl	153+167+168	168						4.78
Biphenyl, dimethyl	167+182	182						5.25
Biphenyl, trimethyl		196						5.74
Benzylquinoline	218	219						4.35

Explanation of the first part of sub-fraction abbreviations: D = DMA; B = BIL; 180 = B180; 500 = B500; A = AC; R = RC; O = OC; H = HMC. Explanation of the second part of sub-fraction abbreviations: A = aromatic
The number indicates the sub-fraction number.

Table A4. Presence of selected resin compounds in active resin sub-fractions of DMA, B180, and B500 oil, respectively, as identified by GC-MS analyses and indicated by bullets. No compounds were identified in the other oils. Tables for these oils are therefore omitted.

Name	Search masses	MW	DR3	DR4	DR5	DR6	DR7	DR8	DR9	SPARC log K_{ow}
Pyrrole	67	67								
Pyrrole, methyl-	80+81	81								
Pyrrole, dimethyl-	94+95	95								
Pyrrole, trimethyl-	108+109	109								
Pyrrole, ethyl-	80+95	95								
Pyrrole, ethyl, methyl-	94+109	109								
Benzopyrrole	90+117	117								
Furan	68	68								
Furan, methyl-	81+82	82								
Furan, dimethyl-	95+96	96								
Furan, trimethyl-	109+110	110								
Furan, ethyl-	81+96	96								
Furan, methyl, ethyl-	81+156	156								
Furan, propyl-	81+110	110								
Pyridine	79	79								
Pyridine, methyl-	93	93								
Pyridine, dimethyl-	106+107	107								
Pyridine, trimethyl-	102+121	121								
Pyridine, ethyl-	106+107	107								
Pyridine, diethyl-	134+135	135								
Pyridine, propyl-	92+121	121								
Pyridine, phenyl-	154+155	155								
Pyridine, diphenyl-	230+231	231								
Pyridine, benzyl-	168+169	169								
Pyridine, 2,6-di-p-tolyl-	259	259								
Pyrazine	80	80								
Pyrazine, methyl-	94	94								
Pyrazine, dimethyl-	108	108								
Pyrazine, trimethyl-	122	122								
Pyrazine, tetramethyl-	136	136								
Pyrazine, ethyl-	107	108								
Pyrazine, propyl-	94	122								
Thiophene	84	84								
Thiophene, methyl-	97+98	98								
Thiophene, dimethyl-	111+112	112								
Thiophene, ethyl-	97+112	112								
Thiophene, diethyl-	125+140	140								
Thiophene, propyl-	97+126	126								
Benzothiophene	134	134								
Dibenzothiophene	184+185	184								
Pyridinamine	94	94								
Pyridinamine, methyl-	80+108	108								
Pyridinamine, dimethyl-	94+122	122								
Pyridinamine, trimethyl-	107+136	136								
Cresol	107+108	107								
Cresol, ethyl-	121+136	136								
Indole	90+117	117								
Indole, methyl-	130+131	131								
Indole, dimethyl-	144+145	145								
Indole, trimethyl-	158+159	159								
Indole, ethyl-	130+145	145								
Indole, phenyl-	165+193	193								

Name	Search masses	MW	DR3	DR4	DR5	DR6	DR7	DR8	DR9	SPARC log K_{ow}
Benzofuran	118	118								
Benzofuran, methyl-	131+132	132								
Benzofuran, dimethyl-	145+146	146								
Benzofuran, ethyl-	131+146	146								
Benzimidazole	118	118								
Benzimidazole, methyl-	131+132	132								
Benzimidazole, dimethyl-	145+146	146								
Benzimidazole, trimethyl-	159+160	160								
Benzimidazole, ethyl-	145+146	146								
Benzimidazole, propyl-	132+160	160								
Benzimidazole, phenyl-	194	194								
Indoline	118+119	119								
Indoline, methyl-	118+133	133								
Naphthalene	128	128								
Naphthalene, methyl-	141+142	142								
Naphthalene, dimethyl-	141+156	156								
Naphthalene, trimethyl-	155+170	170								
Naphthalene, ethyl-	141+156	156								
Naphthalene, propyl-	141+170	170								
Azulene	128	128								
Azulene, trimethyl-	155+170	170								
Azulene, triphenyl-	356	356								
Azulene, dimethyl, ethyl-	169+184	184								
Azulene, dimethyl, isopropy	183+198	198								
Azulene, dimethyl, phenyl-	232	232								
Quinoline	129	129								
Quinoline, methyl-	142+143	143								
Quinoline, phenyl-	204+205	205								
Lepidine	143	143								
Lepidine, ethyl-	170+171	171								
Lepidine, phenyl-	204+219	219								
Naphthol	115+144	144								3.18
Naphthol, methyl-	129+158	158								3.75
Naphthol, dimethyl-	157+172	172								
Carbazole	167	167								
Carbazole, methyl-	180+181	181	●							4.05
Carbazole, dimethyl-	194+195	195		●	●					4.53
Carbazole, trimethyl-	194+209	209		●						5.02
Carbazole, 9-phenyl-	243+244	243								5.43
Benzocarbazole	216+217	217								5.15
Dibenzocarbazole	267+268	267								
Dibenzofuran	139+168	168								
Dibenzofuran, methyl-	181+182	182								
Dibenzofuran, amine-	183+184	183								
Dibenzofuran, phenyl-	244	244								
Acridine	178+179	179								
Acridine, methyl-	192+193	193								
Benzoacridine	229	229								
Indane	117+118	118								
Naphtylamine	143	143								
Hydroxyquinoline	117+145	145								
Anthracene	178	178								
Phenoxazine	154+183	183								
Aminoanthracene /Aminc	165+193	193								
9H-Fluorene-2-carboxylic	207+224	224								
Pyrenecarboxaldehyde	201+230	230								
Benzothiazole	108+135	135								
Benzothiazole, methyl	108+149	149								
Benzothiazole, dimethyl	121+163	163								

Name	Search masses	MW	180R3	180R4	180R5	180R6	180R7	180R8	180R9	SPARC log K_{ow}
Pyrrole	67	67								
Pyrrole, methyl-	80+81	81								
Pyrrole, dimethyl-	94+95	95								
Pyrrole, trimethyl-	108+109	109								
Pyrrole, ethyl-	80+95	95								
Pyrrole, ethyl, methyl-	94+109	109								
Benzopyrrole	90+117	117								
Furan	68	68								
Furan, methyl-	81+82	82								
Furan, dimethyl-	95+96	96								
Furan, trimethyl-	109+110	110								
Furan, ethyl-	81+96	96								
Furan, methyl, ethyl-	81+156	156								
Furan, propyl-	81+110	110								
Pyridine	79	79								
Pyridine, methyl-	93	93								
Pyridine, dimethyl-	106+107	107								
Pyridine, trimethyl-	102+121	121								
Pyridine, ethyl-	106+107	107								
Pyridine, diethyl-	134+135	135								
Pyridine, propyl-	92+121	121								
Pyridine, phenyl-	154+155	155								
Pyridine, diphenyl-	230+231	231		●						4.97
Pyridine, benzyl-	168+169	169								
Pyridine, 2,6-di-p-tolyl-	259	259			●					5.93
Pyrazine	80	80								
Pyrazine, methyl-	94	94								
Pyrazine, dimethyl-	108	108								
Pyrazine, trimethyl-	122	122								
Pyrazine, tetramethyl-	136	136								
Pyrazine, ethyl-	107	108								
Pyrazine, propyl-	94	122								
Thiophene	84	84								
Thiophene, methyl-	97+98	98								
Thiophene, dimethyl-	111+112	112								
Thiophene, ethyl-	97+112	112								
Thiophene, diethyl-	125+140	140								
Thiophene, propyl-	97+126	126								
Benzothiophene	134	134								
Dibenzothiophene	184+185	184								
Pyridinamine	94	94								
Pyridinamine, methyl-	80+108	108								
Pyridinamine, dimethyl-	94+122	122								
Pyridinamine, trimethyl-	107+136	136								
Cresol	107+108	107								
Cresol, ethyl-	121+136	136								
Indole	90+117	117								
Indole, methyl-	130+131	131								
Indole, dimethyl-	144+145	145								
Indole, trimethyl-	158+159	159								
Indole, ethyl-	130+145	145								
Indole, phenyl-	165+193	193								

Name	Search masses	MW	180R3	180R4	180R5	180R6	180R7	180R8	180R9	SPARC log K_{ow}
Benzofuran	118	118								
Benzofuran, methyl-	131+132	132								
Benzofuran, dimethyl-	145+146	146								
Benzofuran, ethyl-	131+146	146								
Benzimidazole	118	118								
Benzimidazole, methyl-	131+132	132								
Benzimidazole, dimethyl-	145+146	146								
Benzimidazole, trimethyl-	159+160	160								
Benzimidazole, ethyl-	145+146	146								
Benzimidazole, propyl-	132+160	160								
Benzimidazole, phenyl-	194	194								
Indoline	118+119	119								
Indoline, methyl-	118+133	133								
Naphthalene	128	128								
Naphthalene, methyl-	141+142	142								
Naphthalene, dimethyl-	141+156	156								
Naphthalene, trimethyl-	155+170	170								
Naphthalene, ethyl-	141+156	156								
Naphthalene, propyl-	141+170	170								
Azulene	128	128								
Azulene, trimethyl-	155+170	170								
Azulene, triphenyl-	356	356								
Azulene, dimethyl, ethyl-	169+184	184								
Azulene, dimethyl, isopropy	183+198	198								
Azulene, dimethyl, phenyl-	232	232								
Quinoline	129	129								
Quinoline, methyl-	142+143	143								
Quinoline, phenyl-	204+205	205								
Lepidine	143	143								
Lepidine, ethyl-	170+171	171								
Lepidine, phenyl-	204+219	219								
Naphthol	115+144	144								3.18
Naphthol, methyl-	129+158	158								3.75
Naphthol, dimethyl-	157+172	172								
Carbazole	167	167								
Carbazole, methyl-	180+181	181	●							4.05
Carbazole, dimethyl-	194+195	195	●	●						4.53
Carbazole, trimethyl-	194+209	209		●						5.02
Carbazole, 9-phenyl-	243+244	243								5.43
Benzocarbazole	216+217	217		●						5.15
Dibenzocarbazole	267+268	267								
Dibenzofuran	139+168	168								
Dibenzofuran, methyl-	181+182	182								
Dibenzofuran, amine-	183+184	183								
Dibenzofuran, phenyl-	244	244		●						-
Acridine	178+179	179								
Acridine, methyl-	192+193	193								
Benzoacridine	229	229								
Indane	117+118	118								
Naphtylamine	143	143								
Hydroxyquinoline	117+145	145								
Anthracene	178	178								
Phenoxazine	154+183	183								
Aminoanthracene /Aminc	165+193	193								
9H-Fluorene-2-carboxylic	207+224	224								
Pyrenecarboxaldehyde	201+230	230								
Benzothiazole	108+135	135								
Benzothiazole, methyl	108+149	149								
Benzothiazole, dimethyl	121+163	163								

Name	Search masses	MW	500R2	500R3	500R4	500R5	500R6	500R7	500R8	500R9	SPARC log K_{ow}
Pyrrole	67	67									
Pyrrole, methyl-	80+81	81									
Pyrrole, dimethyl-	94+95	95									
Pyrrole, trimethyl-	108+109	109									
Pyrrole, ethyl-	80+95	95									
Pyrrole, ethyl, methyl-	94+109	109									
Benzopyrrole	90+117	117									
Furan	68	68									
Furan, methyl-	81+82	82									
Furan, dimethyl-	95+96	96									
Furan, trimethyl-	109+110	110									
Furan, ethyl-	81+96	96									
Furan, methyl, ethyl-	81+156	156									
Furan, propyl-	81+110	110									
Pyridine	79	79									
Pyridine, methyl-	93	93									
Pyridine, dimethyl-	106+107	107									
Pyridine, trimethyl-	102+121	121									
Pyridine, ethyl-	106+107	107									
Pyridine, diethyl-	134+135	135									
Pyridine, propyl-	92+121	121									
Pyridine, phenyl-	154+155	155									
Pyridine, 2,6 diphenyl-	230+231	231			•						4.97
Pyridine, benzyl-	168+169	169									
Pyridine, 2,6-di-p-tolyl-	259	259				•					5.93
Pyrazine	80	80									
Pyrazine, methyl-	94	94									
Pyrazine, dimethyl-	108	108									
Pyrazine, trimethyl-	122	122									
Pyrazine, tetramethyl-	136	136									
Pyrazine, ethyl-	107	108									
Pyrazine, propyl-	94	122									
Thiophene	84	84									
Thiophene, methyl-	97+98	98									
Thiophene, dimethyl-	111+112	112									
Thiophene, ethyl-	97+112	112									
Thiophene, diethyl-	125+140	140									
Thiophene, propyl-	97+126	126									
Benzothiophene	134	134									
Dibenzothiophene	184+185	184									
Pyridinamine	94	94									
Pyridinamine, methyl-	80+108	108									
Pyridinamine, dimethyl-	94+122	122									
Pyridinamine, trimethyl-	107+136	136									
Cresol	107+108	107									
Cresol, ethyl-	121+136	136									
Indole	90+117	117									
Indole, methyl-	130+131	131									
Indole, dimethyl-	144+145	145									
Indole, trimethyl-	158+159	159									
Indole, ethyl-	130+145	145									
Indole, phenyl-	165+193	193									

Name	Search masses	MW	500R2	500R3	500R4	500R5	500R6	500R7	500R8	500R9	SPARC log K_{ow}
Benzofuran	118	118									
Benzofuran, methyl-	131+132	132									
Benzofuran, dimethyl-	145+146	146									
Benzofuran, ethyl-	131+146	146									
Benzimidazole	118	118									
Benzimidazole, methyl-	131+132	132									
Benzimidazole, dimethyl-	145+146	146									
Benzimidazole, trimethyl-	159+160	160									
Benzimidazole, ethyl-	145+146	146									
Benzimidazole, propyl-	132+160	160									
Benzimidazole, phenyl-	194	194									
Indoline	118+119	119									
Indoline, methyl-	118+133	133									
Naphthalene	128	128									
Naphthalene, methyl-	141+142	142									
Naphthalene, dimethyl-	141+156	156									
Naphthalene, trimethyl-	155+170	170									
Naphthalene, ethyl-	141+156	156									
Naphthalene, propyl-	141+170	170									
Azulene	128	128									
Azulene, trimethyl-	155+170	170									
Azulene, triphenyl-	356	356									
Azulene, dimethyl, ethyl-	169+184	184									
Azulene, dimethyl, isopropy	183+198	198									
Azulene, dimethyl, phenyl-	232	232									
Quinoline	129	129									
Quinoline, methyl-	142+143	143									
Quinoline, phenyl-	204+205	205									
Lepidine	143	143									
Lepidine, ethyl-	170+171	171									
Lepidine, phenyl-	204+219	219									
Naphthol (1-Naphthalenol	115+144	144									3.18
Naphthol, methyl- (**1-Naphtl**	129+158	158									3.75
Naphthol, dimethyl-	157+172	172									
Carbazole	167	167		●							3.53
Carbazole, methyl- (3-Methy	180+181	181		●							4.05
Carbazole, dimethyl- (2,6-di	194+195	195		●	●						4.53
Carbazole, trimethyl-	194+209	209			●						5.02
Carbazole, 9-phenyl- (N-phe	243+244	243									5.43
Benzocarbazole (7H-Benzo[	216+217	217		●	●						5.15
Dibenzocarbazole	267+268	267									
Dibenzofuran	139+168	168									
Dibenzofuran, methyl-	181+182	182									
Dibenzofuran, amine-	183+184	183									
Dibenzofuran, phenyl-	244	244			●						-
Acridine	178+179	179									
Acridine, methyl-	192+193	193									
Benzoacridine	229	229									
Indane	117+118	118									
Naphtylamine	143	143									
Hydroxyquinoline	117+145	145									
Anthracene	178	178									
Phenoxazine	154+183	183									
Aminoanthracene /Aminc	165+193	193									
9H-Fluorene-2-carboxylic	207+224	224									
Pyrenecarboxaldehyde	201+230	230									
Benzothiazole	108+135	135									
Benzothiazole, methyl	108+149	149									
Benzothiazole, dimethyl	121+163	163									

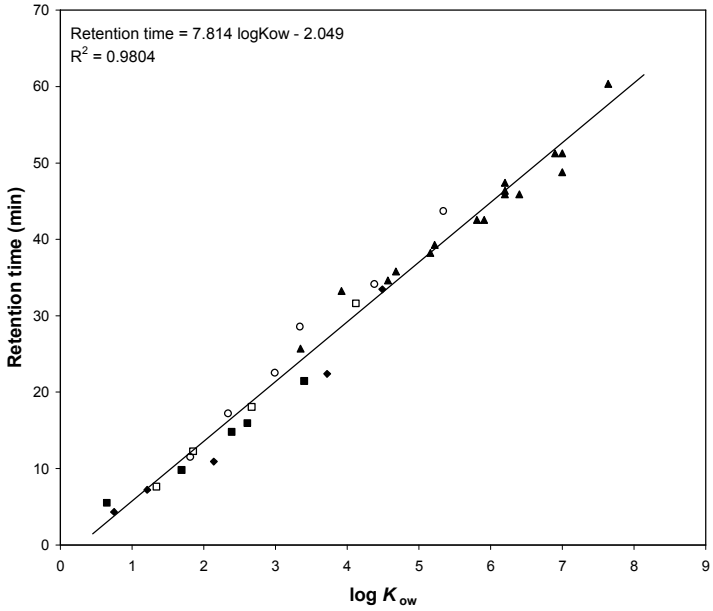


Figure A1: Retention time (min) of 37 aromatic and resin compounds on an Alltima C_{18} column as a function of the compounds' logK_{ow}. Explanation of symbols: ■ pyridines; ▲ PAHs; ♦ pyrroles; o thiophenes; □ furans.

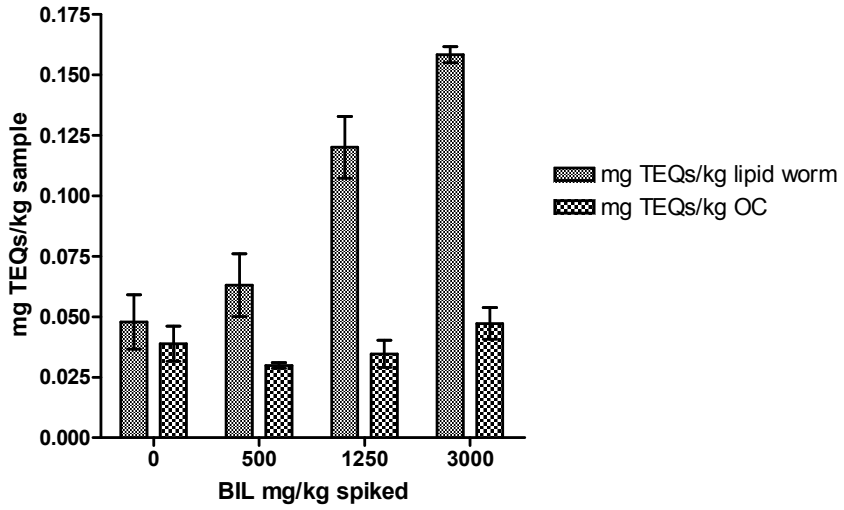


Figure A2: CAFLUX assay responses (mg TEQ/kg sample) to solvent extracts of worms and sediments. The worms were exposed for 4 weeks to the sediment that had been contaminated with different concentrations of Bilge oil (BIL; indicated on the x-axis).

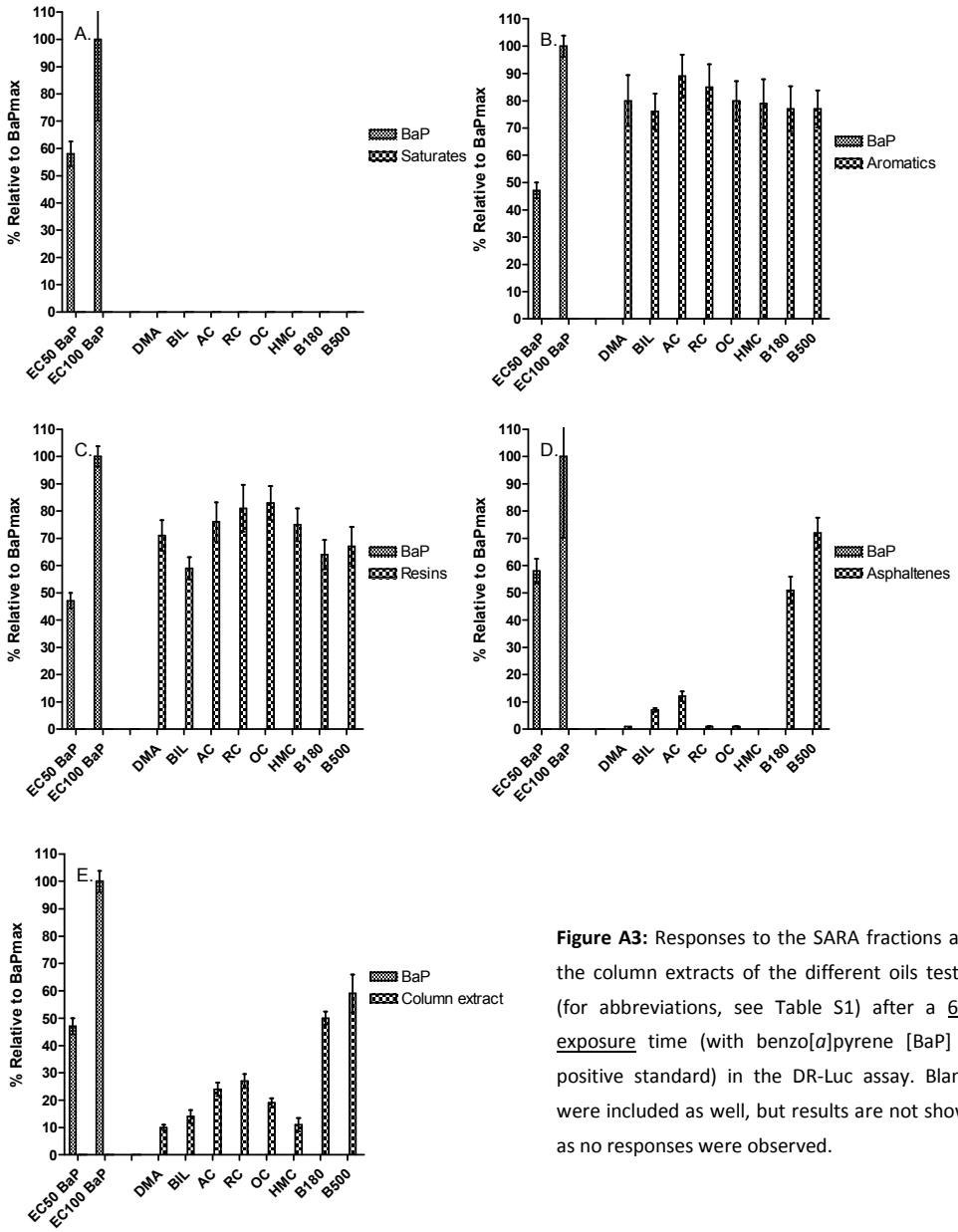


Figure A3: Responses to the SARA fractions and the column extracts of the different oils tested (for abbreviations, see Table S1) after a 6 h exposure time (with benzo[*a*]pyrene [BaP] as positive standard) in the DR-Luc assay. Blanks were included as well, but results are not shown as no responses were observed.

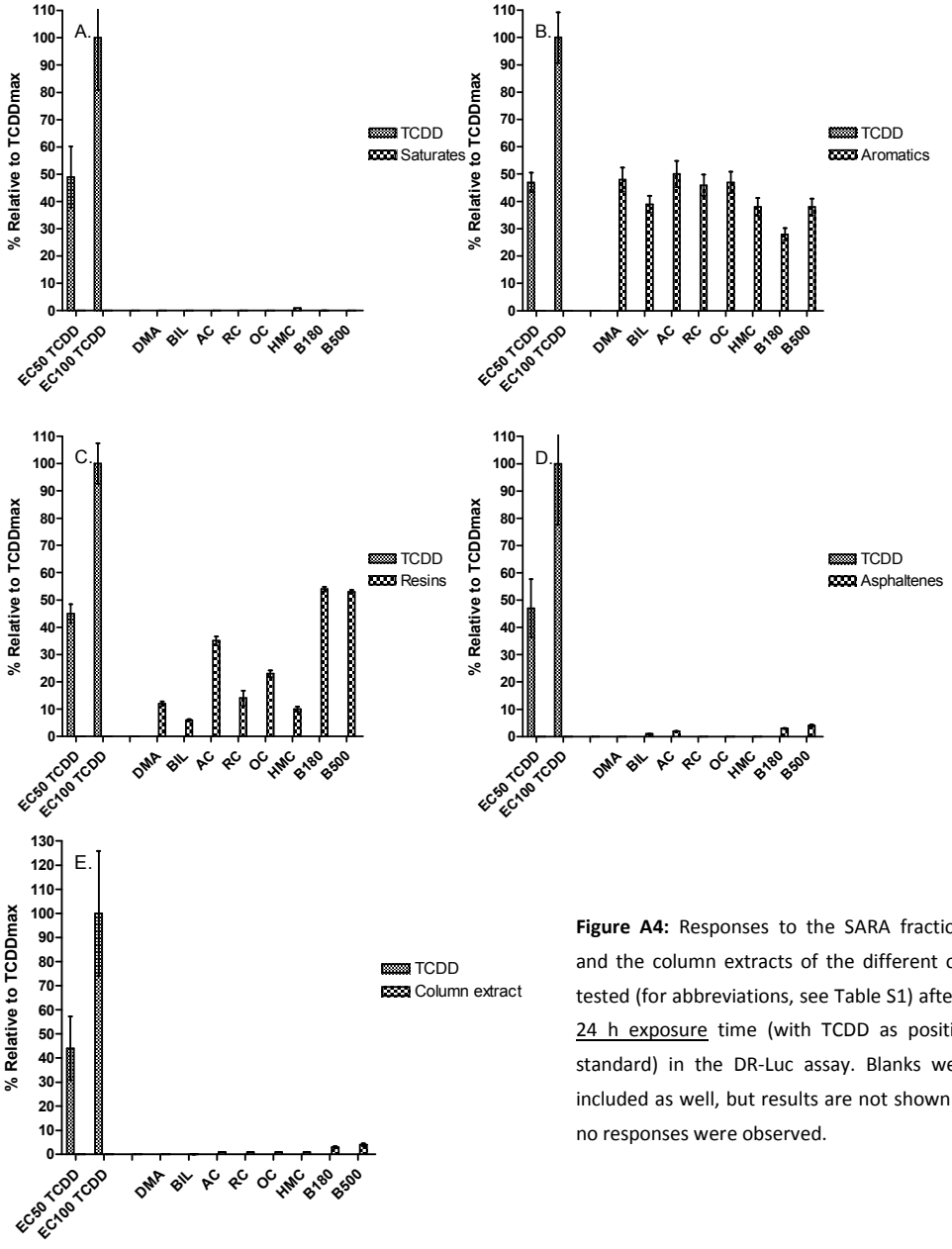
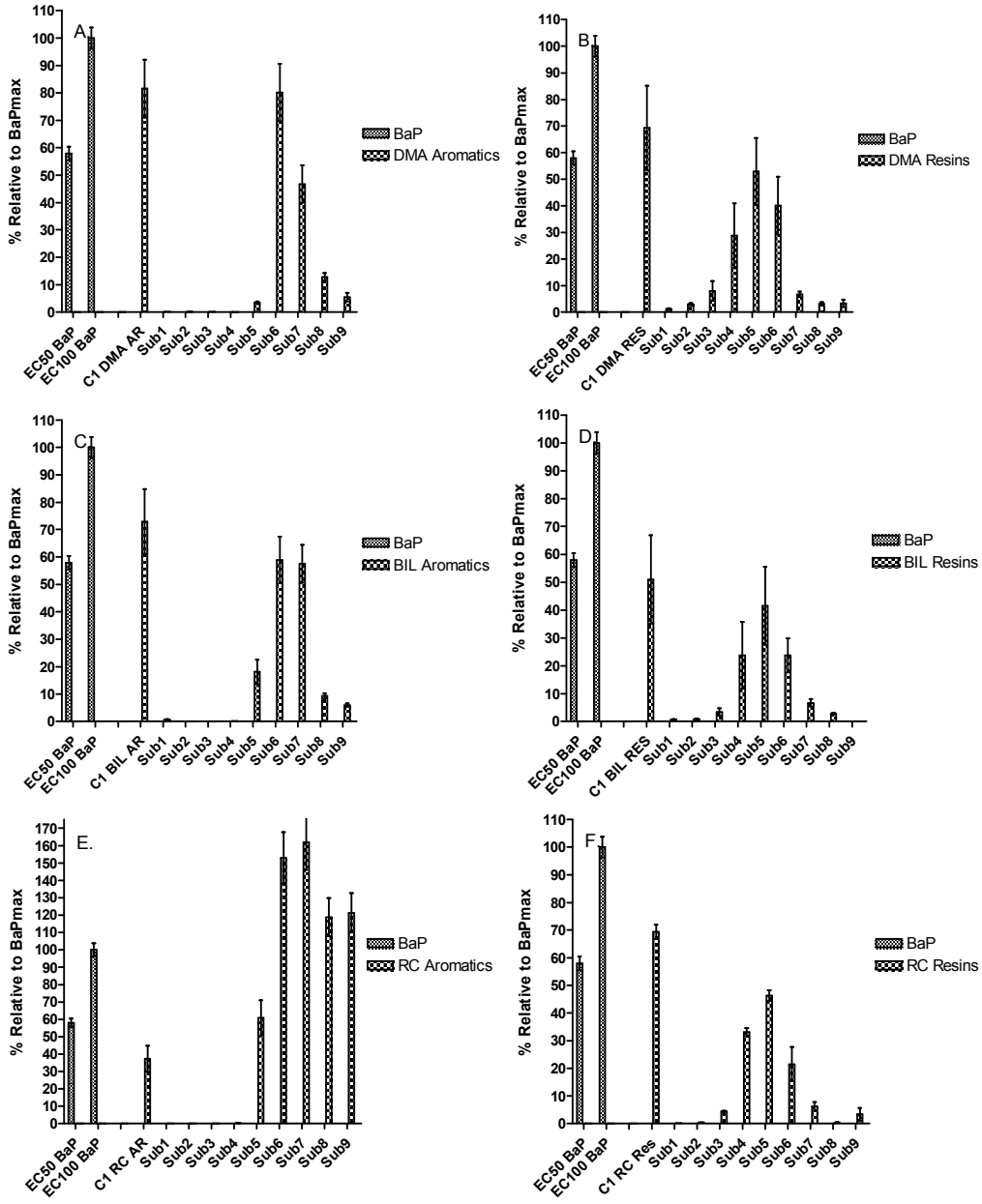
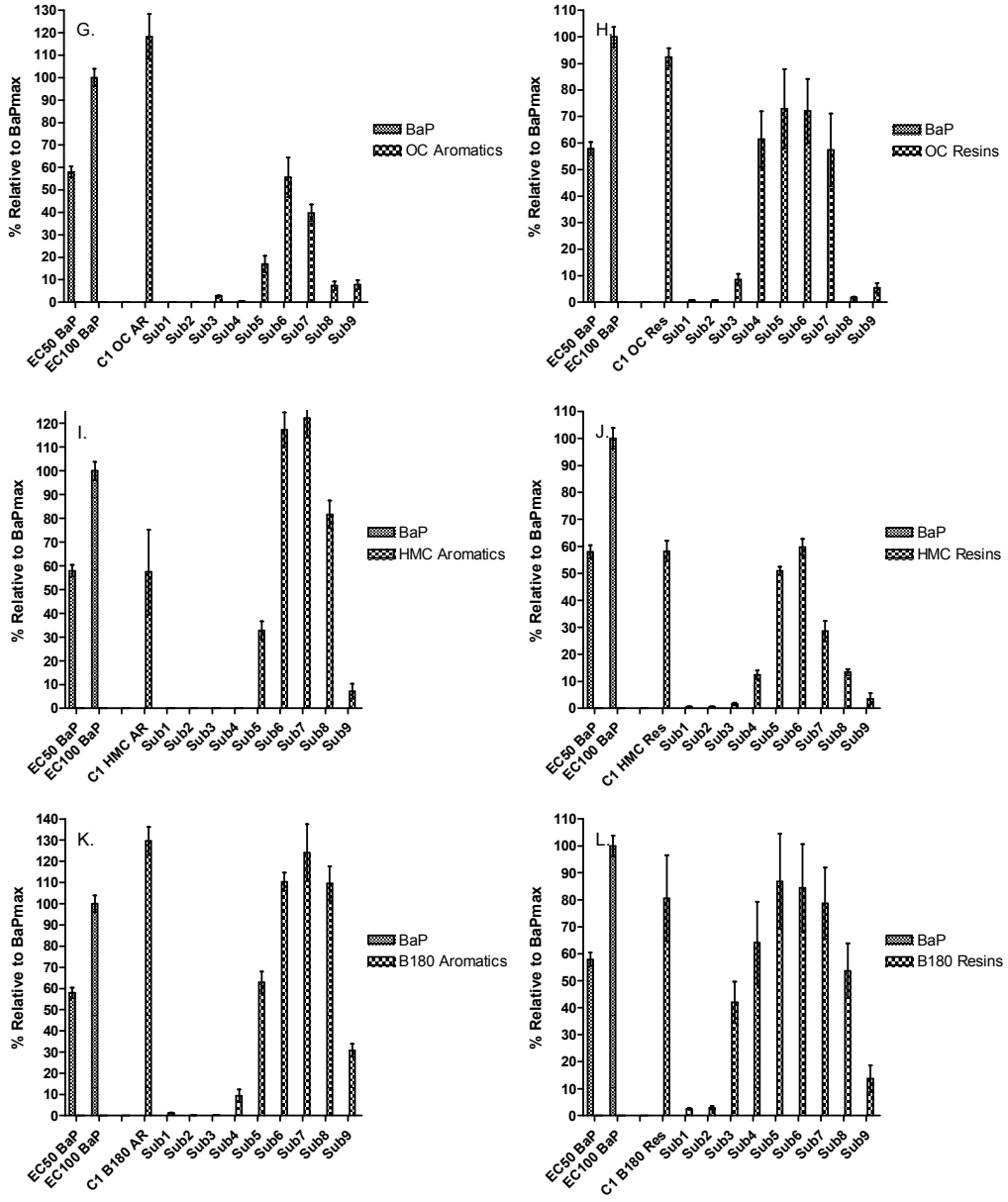


Figure A4: Responses to the SARA fractions and the column extracts of the different oils tested (for abbreviations, see Table S1) after a 24 h exposure time (with TCDD as positive standard) in the DR-Luc assay. Blanks were included as well, but results are not shown as no responses were observed.


A
% Relative to BaPmax
BaP
DMA Aromatics
EC50 BaP
EC100 BaP
C1 DMA AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
B
% Relative to BaPmax
BaP
DMA Resins
EC50 BaP
EC100 BaP
C1 DMA RES
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
C
% Relative to BaPmax
BaP
BIL Aromatics
EC50 BaP
EC100 BaP
C1 BIL AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
D
% Relative to BaPmax
BaP
BIL Resins
EC50 BaP
EC100 BaP
C1 BIL RES
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
E.
% Relative to BaPmax
BaP
RC Aromatics
EC50 BaP
EC100 BaP
C1 RC AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
F
% Relative to BaPmax
BaP
RC Resins
EC50 BaP
EC100 BaP
C1 RC Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9


G.
% Relative to BaPmax
BaP
OC Aromatics
EC50 BaP
EC100 BaP
C1 OC AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
H.
% Relative to BaPmax
BaP
OC Resins
EC50 BaP
EC100 BaP
C1 OC Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
I.
% Relative to BaPmax
BaP
HMC Aromatics
EC50 BaP
EC100 BaP
C1 HMC AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
J.
% Relative to BaPmax
BaP
HMC Resins
EC50 BaP
EC100 BaP
C1 HMC Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
K.
% Relative to BaPmax
BaP
B180 Aromatics
EC50 BaP
EC100 BaP
C1 B180 AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
L.
% Relative to BaPmax
BaP
B180 Resins
EC50 BaP
EC100 BaP
C1 B180 Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9

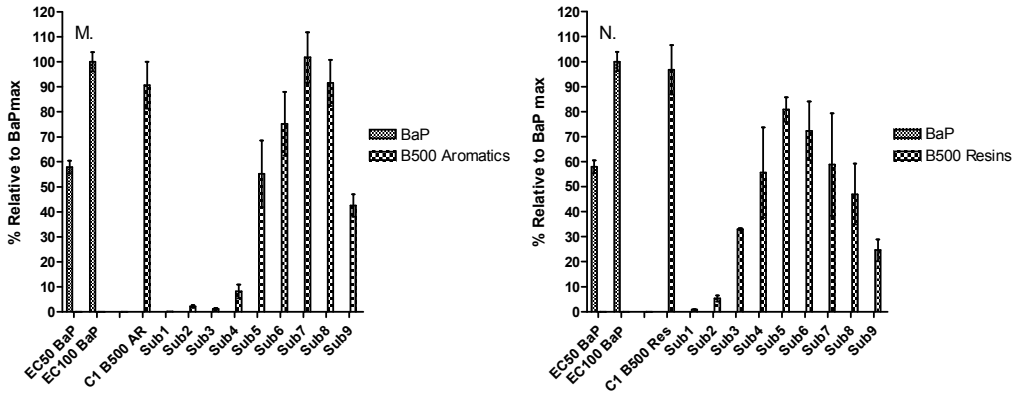
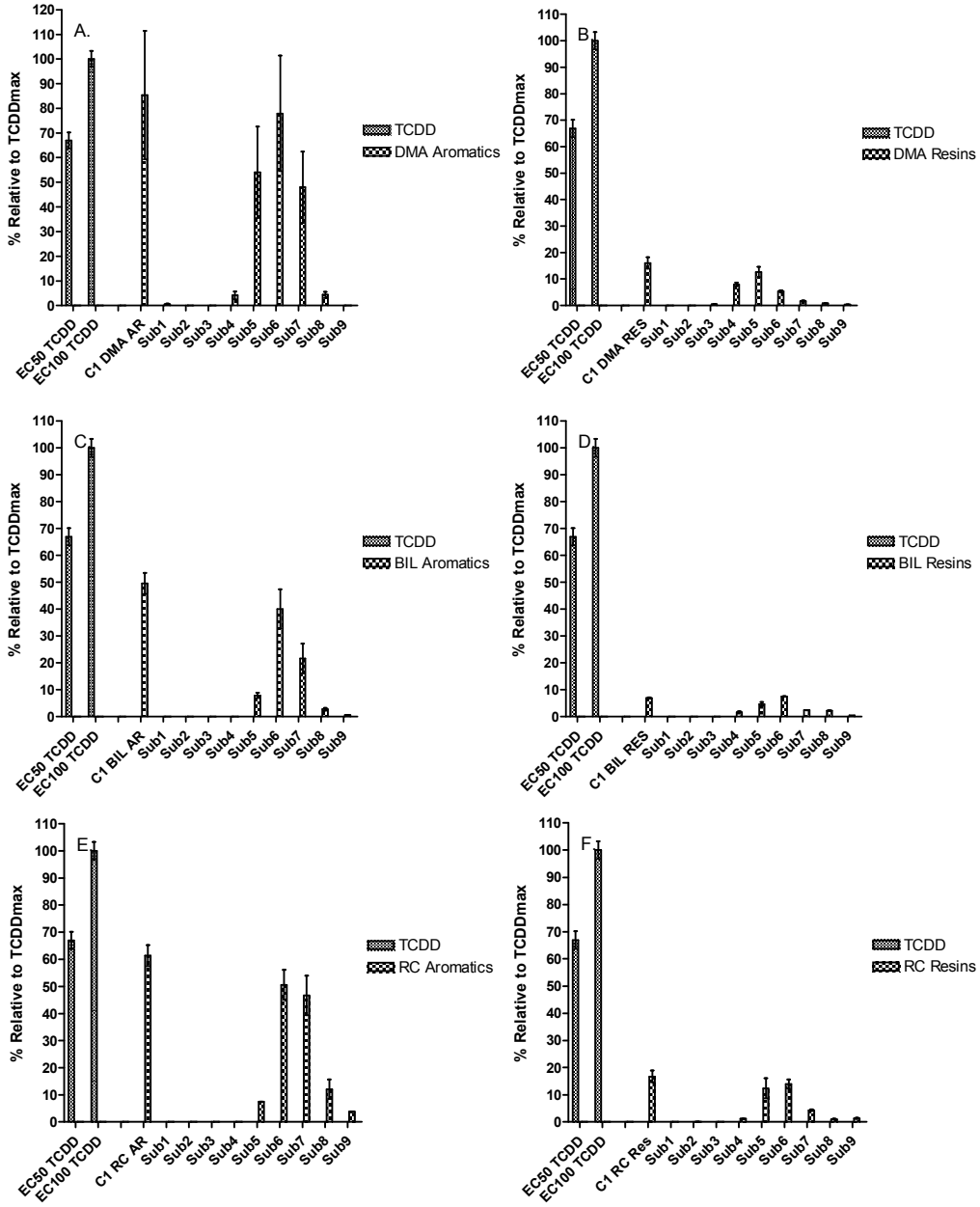
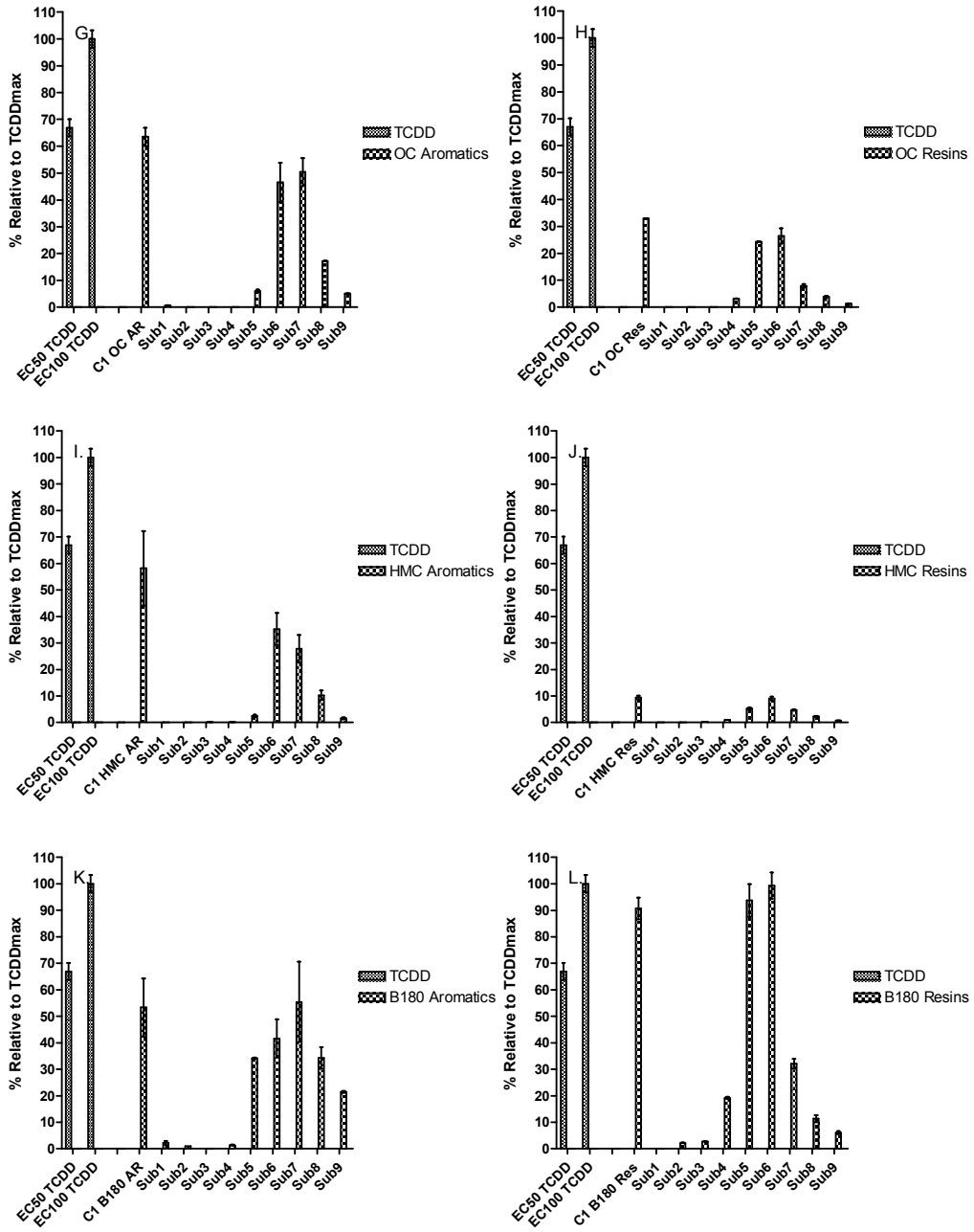


Figure A5: Responses to the aromatic and resin sub-fractions (as obtained by HPLC fractionations) of the different oils tested (for abbreviations, see Table A1) after a 6 h exposure time (with benzo[*a*]pyrene [BaP] as positive standard) in the DR-Luc assay. The C1 bar indicates the response of the original aromatic or resin fraction (before HPLC fractionation). A direct quantitative comparison between the C1 bar and the sub-fraction responses is not possible due to dilutions applied.


A.
% Relative to TCDDmax
TCDD
DMA Aromatics
EC50 TCDD
EC100 TCDD
C1 DMA AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
B
TCDD
DMA Resins
C1 DMA RES
C
TCDD
BIL Aromatics
C1 BIL AR
D
TCDD
BIL Resins
C1 BIL RES
E
TCDD
RC Aromatics
C1 RC AR
F
TCDD
RC Resins
C1 RC Res


G.
% Relative to TCDDmax
TCDD
OC Aromatics
EC50 TCDD
EC100 TCDD
C1 OC AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
H.
% Relative to TCDDmax
TCDD
OC Resins
EC50 TCDD
EC100 TCDD
C1 OC Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
I.
% Relative to TCDDmax
TCDD
HMC Aromatics
EC50 TCDD
EC100 TCDD
C1 HMC AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
J.
% Relative to TCDDmax
TCDD
HMC Resins
EC50 TCDD
EC100 TCDD
C1 HMC Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
K.
% Relative to TCDDmax
TCDD
B180 Aromatics
EC50 TCDD
EC100 TCDD
C1 B180 AR
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9
L.
% Relative to TCDDmax
TCDD
B180 Resins
EC50 TCDD
EC100 TCDD
C1 B180 Res
Sub1
Sub2
Sub3
Sub4
Sub5
Sub6
Sub7
Sub8
Sub9

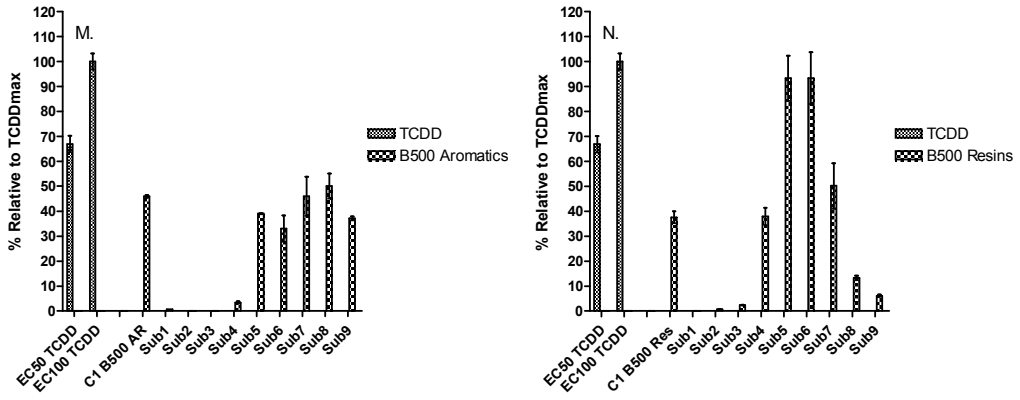
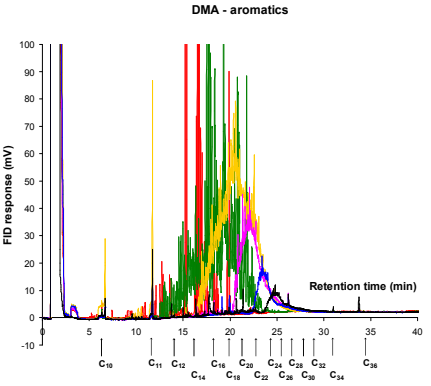
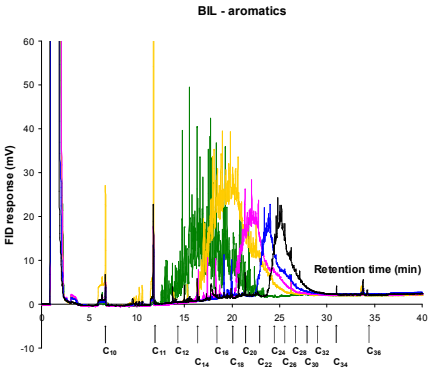
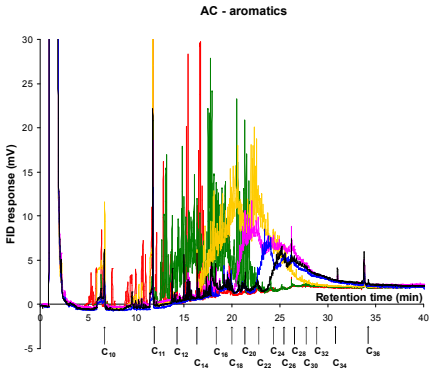
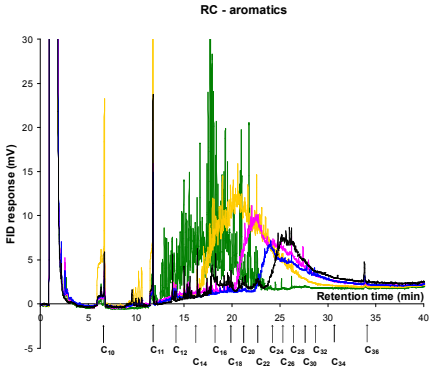


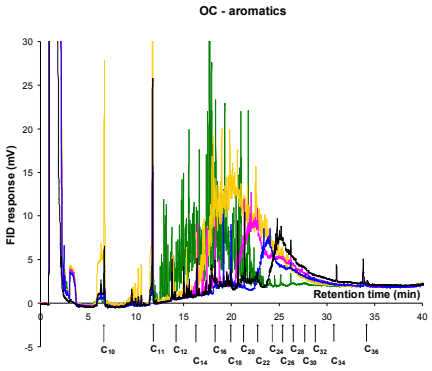
Figure A6: Responses to the aromatic and resin sub-fractions (as obtained by HPLC fractionations) of the different oils tested (for abbreviations, see Table S1) after a 24 h exposure time (with TCDD as positive standard) in the DR-Luc assay. The C1 bar indicates the response of the original aromatic or resin fraction (before HPLC fractionation). A direct quantitative comparison between the C1 bar and the sub-fraction responses is not possible due to dilutions applied.

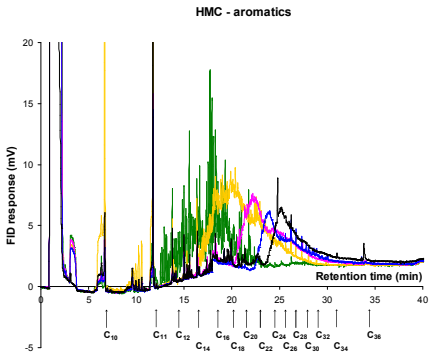

DMA - aromatics
FID response (mV)
Retention time (min)


BIL - aromatics
FID response (mV)
Retention time (min)


AC - aromatics
FID response (mV)
Retention time (min)


RC - aromatics
FID response (mV)
Retention time (min)


OC - aromatics
FID response (mV)
Retention time (min)


HMC - aromatics
FID response (mV)
Retention time (min)

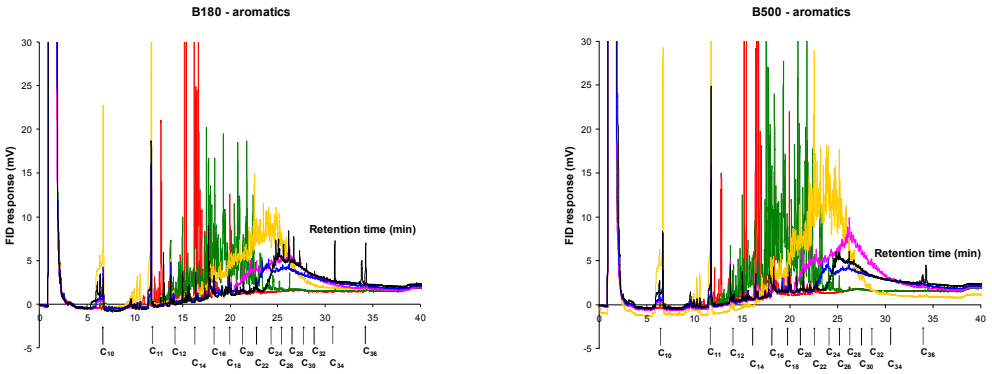
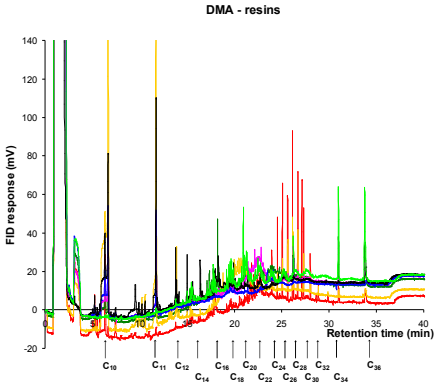
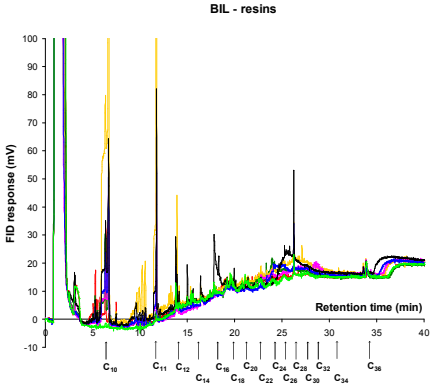
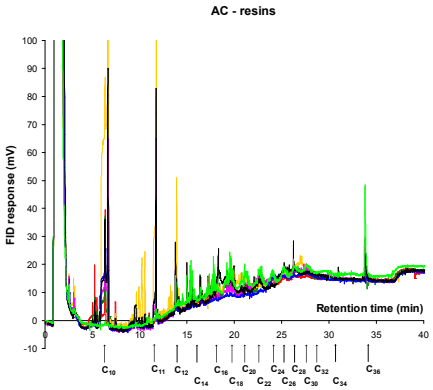
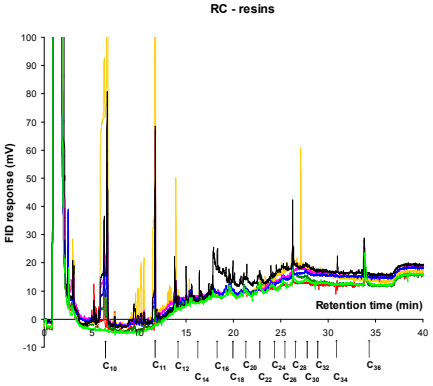


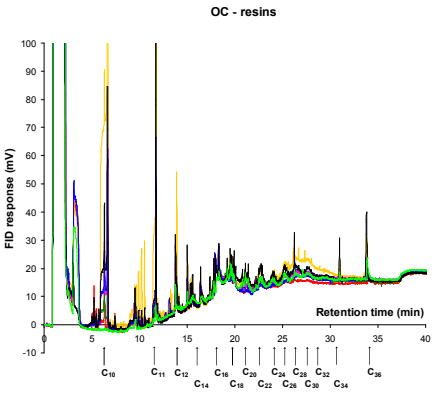
Figure A7. GC-FID chromatograms of the active aromatic sub-fractions of the eight oils tested. Different colors represent different sub-fractions (F-x). Red – F4; dark green – F5; yellow – F6; pink – F7; blue – F8; black – F9. Retention times of linear alkanes are indicated at the x-axes.

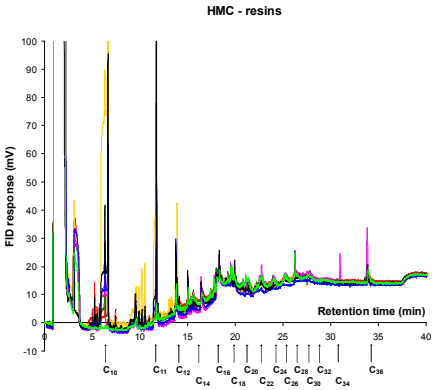

DMA - resins
FID response (mV)
Retention time (min)


BIL - resins
FID response (mV)
Retention time (min)


AC - resins
FID response (mV)
Retention time (min)


RC - resins
FID response (mV)
Retention time (min)


OC - resins
FID response (mV)
Retention time (min)


HMC - resins
FID response (mV)
Retention time (min)

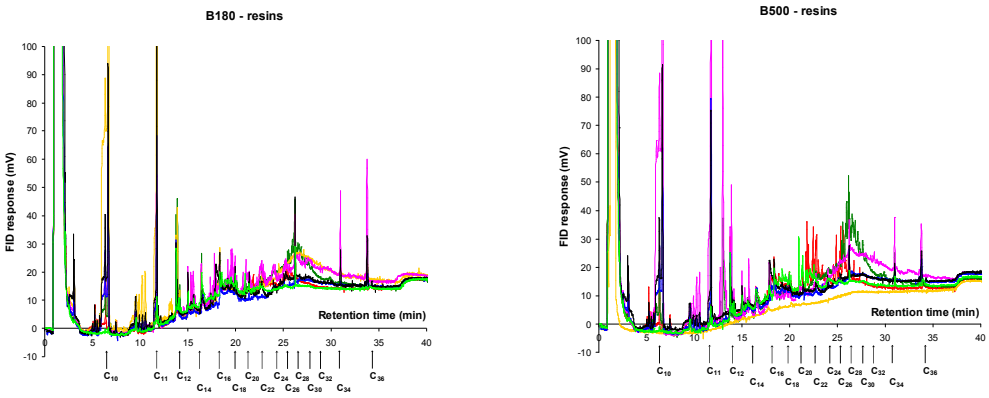


Figure A8: GC-FID chromatograms of the active resin sub-fractions of the eight oils tested. Different colors represent different sub-fractions (F-x). Light green – F3; red – F4; dark green – F5; yellow – F6; pink – F7; blue – F8; black – F9. Retention times of linear alkanes are indicated at the x-axes.

Chapter 6

Summary and general discussion

Abstract

Crude oils and refined oil products are major pollutants of the environment. Large oil spills, such as the recent blowout of Deepwater Horizon in the Golf of Mexico, raise questions on the long term health impact of petroleum hydrocarbons on wildlife and humans. In the environmental risk assessment of oils, narcosis (the disruption of an organism's membrane structure/functioning) is the only mode of action assumed to determine the toxicity of petroleum hydrocarbons. However, several crude oils, refined products, offshore drilling produced waters, and oil-sands process affected water display endocrine disruptive properties in both *in vitro* and *in vivo* systems. Unfortunately, the available information is fragmentary in the sense that only a few oil types have been tested in only one type of *in vitro* assay at a single time point, the causative compounds have not been identified, and the bioaccumulation potential of the *in vitro* active chemicals has not been determined. Therefore, the aims of the research presented in this thesis were to identify the possible endocrine disruptive properties of different, environmentally-relevant crude oils and refined oil products in a battery of *in vitro* bioassays, identify the chemical classes responsible for the observed effects, and link the *in vitro*-observed effects to the hydrophobicity (and thus the bioaccumulation potential) of these chemical classes. This thesis supports the evidence that chronic exposure of organisms to low concentrations of petroleum hydrocarbons might result in specific effects and that narcosis is not the only mode of toxic action. The predicted bioaccumulation potential of the toxic chemicals in oils and their capacity to activate the aryl hydrocarbon-receptor offer a first insight into the classification of these chemicals as potentially bioaccumulative. These findings may help improve the environmental hazard and risk assessment of oils and help prioritize oil spill clean-up activities.

Summary of results

At the time when the research in this thesis was started, little was known about the possible activation of the aryl hydrocarbon receptor (AhR) by crude oils and refined petroleum products. However, there was evidence that oil refineries are an important source of dioxins [1,2], which are known AhR ligands. Moreover, the contamination of the environment with refined products and crude oils, and the public's concern about the possible health effects of oil exposure are widespread [3,4]. These reasons provided ample grounds for starting this research. Furthermore, the work published by Ziccardi et al. (2002) and Jonker et al. (2006) indicated that oils can activate the AhR [5,6]. Therefore, in **Chapter 2** a total of 11 crude oils and refined products (referred to further as oils) were tested for their potential to activate the AhR in a dioxin receptor-mediated luciferase reporter gene assay (DR-Luc assay). By exposing rat hepatoma (H4IIE.luc) cells for 6 and 24 h to different oil concentrations, we screened for easily metabolized compounds (e.g. polycyclic aromatic

hydrocarbon (PAH)-like) and biotransformation-resistant compounds (e.g. dioxin-like), respectively. All oils activated the AhR, especially after 6 h, with responses of several oils exceeding the response of the positive control, benzo[*a*]pyrene (BaP). After 24 h, the responses were lower, indicating the presence of readily metabolized agonists. Several oils, however, still caused high responses after 24 h, also demonstrating the presence of persistent agonists. The responses among refined and crude oils varied from no responses for gasoline and kerosene to high responses for bunker and crude oils. This difference may be explained by differences in the types and concentrations of agonists between the different oils, which, in turn may be explained by the different origins of the oils. Experiments in which oils were tested in combination with the standards demonstrated that oils acted via an antagonistic or additive mode.

In addition to AhR activation, literature indicated that some crude oils and heavy fuel oils display (anti)estrogenic and antiandrogenic activities *in vitro* [7-9]. However, several questions remained unanswered. These questions included whether this observed activity applies only to crude and heavy oils and whether one oil type can induce more than one effect (e.g. one oil type displaying estrogenic and as well androgenic effects). Therefore, possible interactions with the estrogen and androgen hormonal system were investigated in **Chapter 3** and **Chapter 4.** In **Chapter 3** the (anti)estrogenicity and (anti)androgenicity of oils was studied by employing a recombinant yeast stably transfected with human estrogen receptor alpha (ERα), beta (ERβ), or androgen receptor (AR), and expressing yeast enhanced green fluorescent protein. None of the oils tested produced significant estrogenic effects in the ERα assay or androgenic effects in the AR assay. However, all oils were capable of inducing estrogenic responses in the ERβ assay, with several responses being 160% of the maximum induction by the positive control, 17β-estradiol (E2). Based on the lowest effect concentrations, the potencies of oils in all the assays were between 4 and 7 orders of magnitude lower than those of the standard E2 or testosterone (T). The potencies of the individual petrochemical agonists, however, may be very high, because they only make up a very small fraction of the total oil mass (on which the reported potencies were based). Additive, antagonistic, and a synergistic effect were measured in the assays upon co-exposure to a fixed concentration of standard (E2 or T) and increasing concentrations of the oils. These types of combination effects as a result of exposure to mixtures can have deleterious consequences for the developmental phase during organogenesis and reproduction cycles in wildlife [10]. Given that many effects at the cellular level are mediated via the ER and AR, such as cell proliferation and synthesis; and secretion of specific proteins [10], **Chapter 3** also discusses whether the observed effects were receptor-mediated or not. To this end, co-exposures of yeast to the synthetic inhibitors ICI 182,780 (ERβ assay) or flutamide (AR assay), a fixed concentration of standard, and various concentrations of oil were performed. The results suggested that the androgenic effects were receptor-mediated, while the estrogenic effects may be only partially mediated via the receptor.

The yeast bioassays used in this thesis are fast, easy, and robust tools to investigate whether exposure to complex oils may lead to interactions with the hormonal system. However, differences between yeast and mammalian cells in transporters, co-activators, co-repressors, and metabolism stress the need of testing the oils in mammalian assays as well. Therefore, in order to understand if the estrogen receptor activation observed in the yeast assays also occur in mammals, the oils were tested in recombinant mammalian cell assays stably transfected with the human ERα or ERβ, and expressing the luciferase protein (i.e., ERα-U2OS-Luc and ERβ-U2OS-Luc assay). These experiments are discussed in **Chapter 4**. In both assays, all oils, except for two refined and one crude oil, had ER agonist activity. The relative estrogenic potencies of the oils were 6 to 9 orders of magnitude lower than the potency of E2. Upon co-exposure to a fixed concentration of E2 and increasing concentrations of oils, possible additive, antagonistic, and synergistic effects were revealed. Other mechanisms than receptor activation, such as effects on receptor phosphorylation and DNA-receptor complex formation are some of the hypothetical explanations for these types of effects. Other authors suggest the *in vitro* synergistic responses are related to the number of Estrogen Responsive Elements (EREs), their spacing, and the nature of the promoter [11,12]. Additionally, one of the most potent oils, a nautical fuel oil, was also tested in an E-Screen assay with the estrogen-dependent human breast carcinoma cell line (MCF-7). The oil induced cell proliferation up to 70% relative to the maximum induction by E2. At 25 mg/L, the oil's lowest effect concentration, the oil also induced mRNA expression of the estrogen-dependent protein pS2 by a factor of two. These results therefore indicate that oils may induce effects beyond protein secretion and support existing evidence that oils can interfere with the estrogen and androgen signaling pathways.

The *in vitro* assays applied in **Chapters 2, 3**, and **4** support the hazard identification and characterization of these environmentally relevant oils. Although oils are ubiquitous in the environment, the internal exposure of an organism to petroleum hydrocarbons is mainly determined by the potential of these compounds to bioaccumulate [6,13]. The bioaccumulation potential of the petroleum hydrocarbons is expected to depend on their hydrophobicity; although little is known about the fate of specific chemicals in oil causing toxic effects. After an oil spill, in the first 24 h the most volatile compounds such as low boiling point fractions and monoaromatic hydrocarbons will be lost by evaporation. Other compounds with high boiling points, such as PAHs known for their lipophilicity, will persist in the environment and are, therefore, regarded as problematic. Moreover, a high bioaccumulation potential increases the probability of toxic effects and is important in further determining the environmental risks [14]. In **Chapter 5**, the bioaccumulation potential of petroleum hydrocarbons in the aquatic worm *Lumbriculus variegatus* was demonstrated. The bioaccumulated oils were extracted from worms and subsequently the extracts were dosed in the *in vitro* chemically activated fluorescent reporter gene bioassay (CAFLUX) to show their AhR activation effects. Testing of asphaltenic, aliphatic, aromatic, and resin oil fractions in the DR-Luc assays demonstrated that easily metabolized AhR agonists

were present in both the aromatic and resin fractions of all oils. After 24 h exposure, the AhR activation was mostly in the aromatic fraction.

Further fractionation of the aromatic and resin fractions of oils indicated that many different chemicals are responsible for the AhR-mediated effects, as the activity was present in different hydrophobicity fractions, ranging from log K_{ow} of 4 sometimes up to 9. This implies that these chemicals range from moderately to super-hydrophobic and have a moderately to extremely high bioconcentration and bioaccumulation potential.

General discussion

The main objective of the current thesis was to identify specific effects of oils in different recombinant *in vitro* bioassays and to link these specific effects to the bioaccumulation potential of chemical classes from oils. The results of this thesis and their possible application are discussed below in more detail.

Specific in vitro measured effects of oils

In short, the results of **Chapters 2-4** do demonstrate that in the *in vitro* assays used in the current thesis, different oils can activate the AhR, ERα, ERβ, and AR; and have effects on estrogen dependent gene expression and cell proliferation. Although the results suggest that different types of compounds from oil can activate the AhR, one should be careful in concluding that exposure to chemicals that activate the AhR will implicitly mean a dioxin-like toxicity. For instance, it has been postulated that certain chemicals can act as AhR modulators and induce responses that are beneficial (Okey A. and references therein [15]). Furthermore, compounds acting as antagonists for TCDD can possibly prevent downstream events such as the induction of CYP1A1 [15] whose induction is mediated by AhR [16,17]. Induction of this enzyme, as a result of exposure to PAHs, can result in the formation of DNA adducts, which have been linked to induction of toxic effect in marine fish [18]. Still, adverse biological effects can occur in wildlife as a result of exposure to compounds with dioxin-like toxicity [19,20]. Different species have been reported to be affected, including fish, reptiles, birds, and mammals. Adverse effects on reproduction and fertility, the immune system, and development have been observed [20-23].

The compounds acting on the endocrine system can affect sensitive windows of development from the fetal to the adult stage in humans and wildlife. In relation to oil pollution, exposure of fish to petroleum hydrocarbons has been linked to adverse health effects, such as reduced growth and survival [24], increased levels of vitellogenin-like protein in mussels [25], and adverse effects on reproduction in birds [26-28].

The possible modes of action of the endocrine disruptors are through interactions with the ER or AR, therefore affecting protein secretion, steroidogenesis, and crosstalk pathways including, for example, the AhR. Induction of the CYP 1A isoenzyme after AhR

activation can result in the increased metabolism of the E2 [29]. Also certain estrogenic compounds can act as anti-androgens [30]. In general, regarding the compounds that are able to interfere with the endocrine system, the concerns are possible adverse effects including distorted reproductive ability, altered behavior breast and the development of uterus and prostate cancer [31,32].

For several oils, so-called supermaximal effects were observed in **Chapters 2** and **3**. This type of effect has been reported previously in mammalian assays [33-35]. In the case of the yeast assay, to the best of our knowledge, these effects have not been previously reported. The causes of such effects can be explained by the induction of non-receptor mediated pathways [36], the transfection method used, the nature of the promoter, the number of the estrogen receptor elements (EREs) [12], and post-transcriptional effects, such as stabilization effects of certain oil components on the luciferase enzyme [37] that have no biological significance [37].

In general, it is very difficult to relate the toxicity observed in **Chapters 2-4** to specific compounds present in oils. It can be hypothesized that the toxicity is the result of a few potent compounds present in oils at very low levels. Currently, the available analytical techniques can only help to identify specific chemical classes of compounds and not the individual compounds.

Furthermore, although most of the oils were active in the *in vitro* assays used in the current thesis, the results cannot be translated to the *in vivo* situation yet. Different compounds from oils will follow different toxicokinetic pathways, therefore the compounds reaching target cells after transportation through the blood may be different from the ones being active in *in vitro* assays used here. If the compounds from oil are taken up in an organism, the biotransformation pathways will for some of them increase the elimination; however for others the bioactivation. This bioactivation potency is often not included or is represented at lower levels in *in vitro* bioassays.

The use of current in vitro bioassays and data in hazard identification and risk assessment

After the publication of books such as “Silent Spring” by Rachel Carson in the 1960s [38] and “Our stolen future” by Theo Colborn [39] in the mid 1990s, worldwide awareness of and concern about chemicals with potential endocrine disrupting properties has increased. This kind of public attention fueled regulatory agencies from Europe, the United States, and Japan to ensure chemical safety and, therefore, to develop new strategies for assessing chemicals for their potencies to interact with the endocrine system.

In 1997, in a common effort of the Organization for Economic Cooperation and Development (OECD) countries to develop testing guidelines and to harmonize assessment practices, the Endocrine Disrupter Testing and Assessment (EDTA) task force was established. *In vitro* assays, such as the ER, AR, and thyroid hormone receptor binding affinity assays, transcriptional activation AhR recognition binding assays, etc., have been proposed as Level 2

testing out of Level 5 under the "OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals" [40]. Currently, only one *in vitro* assay for detecting estrogenic activity through ERα transcriptional activation has been adopted by the OECD. From the *in vitro* assays, the transactivation assay for detecting (anti)androgenic activities currently awaits full validation. Below several screening strategies for endocrine disrupters chemicals are presented.

In 1998, the Endocrine Disruptor Screening Program (EDSP) coordinated by the US EPA was established under the Federal Food, Drug, and Cosmetic Act (FFDCA) and had as aim "to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or such other endocrine effect" [41]. Accordingly, new *in vitro* and *in vivo* bioassays assessing whether a chemical in both humans and wildlife can interact with the estrogen, androgen, and thyroid systems were developed, standardized, and validated. The testing of chemicals will be performed in a two-tiered approach. Both *in vitro* and *in vivo* bioassays are included in Tier 1, covering modes of actions, such as ER binding and transactivation, AR binding, and effects on steroidogenesis, and effects along the hypothalamic-pituiatry-gonada/thyroidal axes [41]. The data obtained from Tier 1 are used solely as indicative of the chemical's interference with the endocrine system and the tests are regarded as complementary. By using a weight-of-evidence approach, a decision is made whether the tested substance has the potential to interact with the endocrine system and accordingly, if additional testing should be performed under Tier 2 for detecting any adverse effects that can occur in animals [42]. In 2007 The ToxCast^{TM} screening was launched by U.S. EPA in order to screen untested environmental contaminants for potential toxicity based on bioactivity profiling. This program uses not only high-throughput *in vitro* screens, but also computational chemistry and toxicogenomic technologies. It aims to prioritize chemicals needing toxicity testing. Given that endocrine disruption is a mode of action resulting in toxicity, the results obtained with US EDSP Tier 1 will be compared with those of ToxCast for understanding the potential endocrine activity of one chemical [43].

The new European Union (EU) chemical legislation, REACH (Registration, Evaluation and Authorization of Chemicals), supports the use of *in vitro* bioassays, provided that the tests are suitable, which, in this context, means that these assays have been validated or meet the European Centre for the Validation of Alternative Methods (ECVAM) criteria for entering the pre-validation process [44]. Strictly speaking, the registration under REACH does not require the inclusion of endocrine activity of a substance. Nevertheless, the competent authority can require this type of information from a registrant during the evaluation process.

In case the endocrine disruptive properties of petroleum hydrocarbons should be further assessed, the results of the present thesis can be used as weight of evidence and, together with the results of other tests at this level (Tier 1 or Level 2), be used to prioritize certain oils for higher Tier and Level testing. It should be acknowledged that these *in vitro*

assays as such cannot be used to classify a chemical as endocrine disrupter in wildlife, as binding to a hormone receptor *in vitro* does not necessarily mean that the chemical sufficiently reaches and (in)activates the receptors to cause endocrine disruption *in vivo* . Instead, should the assays used prove to have high data significance (reliability and repeatability); they can be valuable tools in the overall process of assessment of endocrine disruption [42].

The use of *in vitro* assays together with chemical measurements has been recently advocated to be used in the impact assessments and monitoring programs of oil spills [45]. The mammalian and yeast reporter gene bioassays employed in the current thesis are well-developed, specific, rapid, sensitive, and widely used for screening of compounds [46-49]. Given that the results of one assay cannot be used for identification of other types of effects, the application of multiple assays should be regarded as complementary in an effort to understand different modes of action through which chemicals can act. Although biologically more simple than mammalian cells, yeast cells are more resistant to contaminants, easy to culture, and the assays in which they are used are easy to perform and inexpensive. In the context of oil pollution, these assays can be used as exposure assessment tools for an early and fast screening in assessing the level of contamination of environmental compartments and biological samples. Therefore, for initial exposure assessment after an oil spill, the tests applied in the current thesis or the alternative *in vitro* assays suggested by Martínez-Gómez *et al*., should be applied [45]. Furthermore, these batteries of *in vitro* assays covering multiple modes of actions have to be used in conjuncture with *in vivo* ecotoxicity test assays, covering different trophic levels. However, depending on the climate where the spills occur, the selection of the sensitive species used for monitoring should be adapted. Another beneficial application of the assays can be for a fast assessment of any toxic effects of possible dispersants used as remediation techniques after a spill.

Bioaccumulation potential of petroleum hydrocarbons

The results presented in **Chapter 5** have proven the bioaccumulation potential of petrochemicals and also confirmed that not only aromatics can cause toxicity, as previously suggested [50,51]. More polar compounds (NSO), such as for example alkyl phenols, quinolines, thiophenes, acridines, can also contribute to the observed toxicity of petrochemicals [51]. Moreover, several biotic (i.e. species, age, metabolic, elimination rate, etc) and abiotic (i.e. water temperature, pH, salinity, chemical's bioavailability) factors can influence the bioaccumulation process of oil components and their toxicity [14]. This implies that, for example, for one chemical the bioaccumulation potential can differ depending on the environmental conditions and species' sensitivity. Therefore, the results of **Chapter 5** can be regarded as a worst-case scenario. For example, it has been demonstrated that the concentration of petroleum hydrocarbons decreases within 1 month if the exposed organism is placed in clean water [52]. Also due to the activity of detoxification enzymes, the internal

concentrations in biota can change, provided that uptake of petroleum hydrocarbons is not a continuous process. Furthermore, the cytochrome P450 detoxification system differs between species and between the members of the same species [52]. Anyway, compounds accumulating via food and being resistant to metabolism or having a low metabolic degradation are posing a threat to birds and mammals [14,53] given their biomagnification potential. Although the work presented here has given important insights into the hazards of oils, due to the large number of variables mentioned above, risk assessment of oils still remains difficult, all the more because their toxicity can change in time, as will be discussed below.

Does the toxicity of oils change with time?

After its release into the environment, oil will undergo transformation through a series of physical, chemical, and biological processes that will occur during different timescales, ranging from hours to years. The environmental conditions (i.e. temperature, wave energy, and wind speed) at the place of the spill and a series of chemical and biological transformations will determine the fate of oil and, implicitly, its ecological risks [52]. The oil will be spread, stranded onshore, disperse, form emulsions, sorb to particles, sedimentate, and bind to particles. Evers *et al*. [52], argue that the ecological risk towards birds and mammals, littoral and coastal marshes flora and epifauna, fish and benthic organisms, and sediment fauna will occur through floating, stranded, dispersed oil and oil on tidal flats, respectively. Evaporation, dissolution, (photo)chemical reactions, and microbiological degradation, collectively known as weathering, can change the composition of the oil and arguably its toxicity. Evaporation is a very fast process by which most volatile compounds (low boiling point fractions) will evaporate within 24 h after a spill [54], and within the first 10 days of the spill, the C10-C15 n-alkanes can evaporate as well [55]. Dissolution is a slower process, which ensures that compounds with high aqueous solubility will not be present in weathered oil. Instead, weathered oil will be enriched with n-alkanes, alkenes, cycloalkenes, and PAHs having a high molecular weight and low solubility. Compounds such as PAHs can be degraded; however this process depends on their molecular size and the oxygen availability. Moreover, through weathering, the bioavailability of certain compounds can be reduced through the formation of tar balls (’inclusion’ or stronger sorption of certain compounds [56]) and sorption of petroleum hydrocarbons to sediments. Given these mentioned processes that are affecting the oil composition and its physical state, the toxicity will change as well. Indeed, it has been demonstrated that the specific toxicity of oil can change upon weathering. For example, reduced or no AhR activation was observed for 2 year-weathered diesel, bunker, and crude oil upon exposure in the Dioxin Receptor Chemically Activated Luciferase Gene Expression (DR CALUX) assay [6].

Also *in vivo* toxicity may change upon weathering. Under laboratory conditions, Neff *et al*. [57] studied how the toxicity of three crude oils and one diesel fuel oil towards

tropical/subtropical and temperate marine organisms (fish, shrimp, sea urchin larvae) changed as the oils weathered by evaporation. The toxicity of the water-accommodated fractions differed between oils and species and the authors attributed the toxicity to monoaromatic hydrocarbons (MAHs), PAHs, and phenols. In the case of diesel fuel oil, it was indicated that other compounds (e.g. resins) were contributing to the toxicity [57].

In case of the Exxon Valdez spill (Alaska, 1989), however, effects of the oil on wildlife were still present twenty years after the spill. The local conditions caused the oil to persist in this environment, e.g. through the formation of a "mousse", percolation of the oil into fine-grained beaches, and little action of oil-degrading microorganisms [58]. Most of the oil was present in the subsurface sediments and reportedly high concentrations of high molecular weight PAHs remained [59]. Some scientists support the concept that oil sequestered in the subsurface sediments presents no ecological risk to foraging wildlife due to their distribution [60] and because of the decreased bioavailability of PAHs to intertidal plants and animals [61]. On the other hand, increased levels of hepatic EROD activity in harlequin ducks collected from the area twenty years later indicated that these birds are continuously exposed to residues of the spill [62]. Also, exposure of herring larvae to low concentrations of residual oil from the Exxon Valdez spill has been linked to morphological and genetic damage [63].

In other ecosystems, however, the persistence of oil can be low. This was for instance reported for the Nakhodka oil spill on the coast of Japan, where the heavy bunker oil spilled disappeared within three years after the spill. The oil was reported to remain on coasts of bedrock and boulder or pebble, and was removed rapidly from coasts of gravel, sand, and man-made structures [64]. Furthermore, the toxicity of the oil decreased in time, as the organisms from the polluted area have recovered after the spill [65].

In conclusion, the toxicity of oils generally can be assumed to decrease in time. The rate of decrease and the toxicity of the weathered oil will be mostly dependent on the initial composition of the oil, the environmental conditions at the site of the spill, and the presence of oil degrading microorganisms.

Challenges when working with oils

Working with oils for research purposes is not an easy job for many reasons. Before commencing with testing the oils in the *in vitro* bioassays, research was done on how previous studies dosed oils in bioassays. From the papers examined, it was noticed that for different oil types (gasoline, jet fuel, diesel, crude oil, residual oils), solvents such as methanol, ethanol, cyclohexane, and dimethylsulfoxide (DMSO) were used [5,7-9,66]. For example, Arcaro *et al.* [8] mentioned dissolving residual and crude oils first in cyclohexane followed by extraction with DMSO, or by just dissolving the oils in DMSO and applying centrifugation for clarifying the obtained solutions. Ziccardi *et al.* dissolved gas, diesel, jet

fuel, crude, and heavy fuel oils either in methanol, or first in hexane and then in methanol [5].

Before preparing solutions of crude and heavy oils for dosing in *in vitro* assays, assumptions should be made on whether the chemicals in the oils will be taken up directly through the skin of pelagic organisms or via other uptake routes (e.g. food and ingestion of sediments). When assuming the former case, it is advisable to dose only the water-accommodated fractions (WAFs) of oils, in other words the water soluble fraction of oil. When assuming the latter case, there are several requisites that have to be fulfilled. First, a solvent that dissolves all the components of oils should be used. It is not advisable to use centrifugation or filtration for removing the undissolved components, as toxic effects induced by the water-insoluble fraction may be missed. Another method is to use a 'solvent bridge'. Here, oil is first completely dissolved in a solvent, which is immiscible with water, and then mixed with another solvent, a carrier solvent, which is miscible with water. The carrier solvent needs to be miscible in water to dose the oil into the aqueous buffer solutions used in *in vitro* assays (i.e. the cell culture medium). Moreover, the carrier solvent should show no biological activity in the assay. In this thesis, the above aspects were accounted for by using toluene, a solvent capable of completely solubilizing even the heavy oils. Next, the dilutions of oils where prepared by using ethanol, a solvent miscible with toluene and culture medium, hence fulfilling the above-mentioned requirements.

Due to the hydrophobic character and poor solubility of oils, testing high concentrations of oils in *in vitro* assays can result in the formation of a floating oil film on the surface of the cell culture medium. In case the assay medium is not removed, results may be biased when spectrophotometrical absorption measurements are applied. Therefore, it is recommended that any interference is checked for by control experiments, as was done in **Chapter 2**.

The composition of refined and crude oils is very complex. In addition to thousands of unresolved hydrocarbons, crude oils often contain sulfur. This element is highly cytotoxic and precautionary (desulfurizing) measures should therefore be taken in order to avoid cell death that may bias the final results (see **Chapter 2**). Oils contain a wide array of hydrocarbons varying significantly in volatility and hydrophobicity. Volatile compounds (e.g. monoaromatic hydrocarbons) in oil [67] may evaporate out of the well plate of an *in vitro* assay [68-70]. Furthermore, in *in vitro* assays using 96-well plastic plates, it is expected that particularly hydrophobic hydrocarbons will sorb to the plastic and bind to the assay medium's serum proteins, in addition to partitioning into the cells. Kramer *et al.* demonstrated that in case of PAHs with a log K_{OW} between 3 and 6, the presence of serum proteins will significantly reduce the free concentration of PAHs in the medium [71]. Moreover, there is a linear relationship between the log K_{OW} of PAHs and sorption to plastic, binding to serum constituents, and partitioning in the cells. The actual free concentrations of PAHs were estimated to be 0.5-7% in a typical *in vitro* assay [71]. The low free concentrations of compounds in the medium consequently reduce the concentration available for uptake in the cells and implicitly have an

influence on the final results (e.g. EC50s). In general, one would expect that expressing effect concentrations of oils as nominal exposure concentrations leads to an underestimation of the actual toxicity. The results presented in this thesis as such could be considered as a "best case scenario", as they potentially reflect an underestimation of the actual effects.

Given the oils' complex composition of sometimes 100,000 chemicals, one should understand that it is impossible to estimate to which individual compounds the cells will be exposed. Therefore, the final measured effects are also the results of mixture toxicities and not a single chemical. Furthermore, the final effects will be influenced by the capabilities of compounds to pass the biological membranes of mammalian and yeast cells. The plasma membranes of both yeast and mammalian cells are lipid bilayers. It is expected that in case of yeast cells, the transport of compounds through the cell wall can be a limiting (i.e. highly packed polysaccharides around plasma membrane) factor; however, compounds with molecular weight below 620 g/mol have been demonstrated to pass through isolated cell walls [72]. Therefore, compounds such as PAHs can easily be transported across the membranes, however high molecular weight compounds such asphalthenes probably cannot.

Taken together, the above examples demonstrate that working with oils poses not only practical challenges, but also several ones related to the data interpretation. The final results may therefore depend on the test conditions and as well as the properties of the (chemicals present in) oils. Note that the same applies to testing oils in *in vivo* experiments (where oils can e.g. cause smothering of organisms at high concentrations or oxygen depletion during testing, thereby producing false positive results), but this type of work is beyond the scope of the present thesis.

Future perspectives and concluding remarks

With the specific *in vitro* assays applied in the current thesis, it has been demonstrated that in addition to narcosis, refined and crude oils also contain chemicals that can cause specific toxic effects. The studies applied here give point towards the possible endocrine disruptive properties of oil. However, for regulatory acceptance, these oils should be tested, for example, in the validated Tier 1 assays from US EPA. After a spill, the *in vitro* assays accompanied by analytical methods can be used to determine whether exposure of biota to different classes of petroleum chemicals has occurred and as well monitor in time their presence in environmental compartments and biological samples. The bioassays can not only be applied after a spill, but may also be used to understand the impact of other chemicals on the toxicity of oil (e.g. dispersants) and to monitor the recovery of an ecosystem over time. Nevertheless, the *in vitro* assays have to be validated against *in vivo* field data. In validating *in vitro* data against *in vivo* field data, biokinetics models can be used to extrapolate *in vitro* effect concentrations to *in vivo* doses [73,74].

In the current research, it has been shown that the responsible chemicals for at least AhR activation effects are not only aromatics but also polar compounds. To date, the identification of the exact chemicals is impossible; however, most of the chemicals activating the receptor are in the high boiling point fractions, which are known for their hydrophobicity. Therefore, one can assume they are bioaccumulative chemicals, potentially leading to high internal concentrations of these chemicals in organisms, as was confirmed by bioaccumulation tests with aquatic worms [75]. Nevertheless, in the environment, oils can weather, causing some petroleum hydrocarbons to become less bioavailable [6]. Moreover, oils may itself function as a separate sorbing phase for these chemicals [76].

From the specific receptor-mediated effects perspective and based on the current data, a provisional classification of hazardous oils can be made with DMA and bunker oils being the most toxic ones followed by the crude oils. Gasoline and kerosene do not pose an environmental hazard through specific toxic effects, as little or no toxicity was observed in the *in vitro* assays.

According to the results presented in the last chapter of this thesis, oils can potentially bioaccumulate in biota. However, to adequately classify a particular oil or oil fraction as 'Persistent, Bioaccumulative and Toxic' (PBT), according to EU standards, the assessment criteria provided in Annex XIII of the REACH Regulation should be adhered to [77].

Given the high repeatability and reliability of the bioassays used in the current thesis, the data presented can be used as weight-of-evidence by regulatory agencies for assessing the endocrine disrupting potential of oils and therefore, help improve their hazard assessment.

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Vol ANNEX XIII Criteria for the identification of Persistent, Bioaccumulative and Toxic substances, and very Persistent and very Bioaccumulative substances.

Nederlandse samenvatting

Een wereldwijd toenemende vraag naar olie heeft geleid tot een toenemende exploitatie van zowel offshore als inlandse bronnen voor deze olie. De onvermijdelijke blootstelling van verschillende delen van het milieu, zoals water, sediment, grond en lucht, komt vooral door (onbedoelde) ongelukken zoals gebreken in de extractie- en transportsystemen (pijpleidingen), lekkages van tankstations, jachthavens en bunker schepen. De opzettelijke vervuiling daarentegen komt vooral door het dumpen van afval olie uit schepen en auto's.

Gezien het feit dat tijdens een olieramp, zoals de Exxon Valdez of Deepwater Horizon, miljoenen liters ruwe olie vrijkomen in het milieu, en gezien de complexe samenstelling van deze olie, meestal met vele duizenden organische en anorganische stoffen, kan men zich afvragen wat de lange termijn gevolgen zijn van deze petroleum koolwaterstoffen (dat wil zeggen stoffen met C en H atomen) op de gezondheid van mens en natuur. In de milieu risicobeoordeling van oliën is narcosis (de verstoring van de lipiden membraan van een organsime) de enige manier waarvan wordt aangenomen dat het de toxiciteit van petroleum koolwaterstoffen kan bepalen. Echter, een aantal ruwe oliën, geraffineerde producten, water gebruikt bij offshore boren, en het water gebruikt bij teerzand olie winning processen, hebben endocrien verstorende eigenschappen in zowel *in vitro* als *in vivo* systemen. Helaas is de beschikbare informatie slechts fragmentarisch in die zin dat slechts een paar olietypes zijn getest in slechts één type *in vitro* test op maar een enkel tijdstip. Ook is niet vastgesteld welke verbindingen deze verstoringen veroorzaken en wat het vermogen tot bioaccumulatie van de *in vitro* actieve chemicaliën is. Daarom is het doel van dit proefschrift om de mogelijke endocrien verstorende eigenschappen van 11 verschillende, milieu relevante ruwe oliën en geraffineerde olieproducten in een serie van *in vitro* bioassays vast te stellen, de chemische klassen die verantwoordelijk zijn hiervoor te identificeren en de *in vitro* waargenomen effecten te verbinden met de hydrofobiciteit (en dus de bioaccumulatie) van deze chemische klassen.

In **Hoofdstuk 2** zijn de cytotoxiciteit en de aryl koolwaterstof receptor (Ahr) agonistische activiteiten van 11 ruwe oliën en geraffineerde olieproducten bepaald in de *in vitro* 3-(4,5-dimethylthiazool-2-yl) -2,5-diphenyltetrazolium bromide (MTT assay) en de dioxinereceptor gemedieerde luciferase reporter gen assay (DR-Luc). Door het blootstellen van ratten hepatoom (H4IIE.luc) cellen voor 6 en 24 uur aan verschillende concentraties olie, hebben we respectievelijk gescreend voor gemakkelijk te metaboliseren verbindingen (bv. polycyclische aromatische koolwaterstoffen (PAK)-achtige stoffen) en biotransformatie resistente verbindingen (bv. dioxineachtige stoffen). Alle oliën activeerden de AhR, vooral na 6 uur, en de respons van verscheidene oliën was zelfs hoger dan de respons van de positieve controle, benzo [a] pyreen (BaP). Na 24 uur was de respons veelal lager, wat wijst op de aanwezigheid van gemakkelijk te metaboliseren agonisten. Echter, verscheidene oliën hadden na 24 uur nog steeds een hoge respons wat wijst op de aanwezigheid van persistente agonisten. De response tussen de geraffineerde en de ruwe oliën varieerde van geen respons

voor benzine en kerosine tot een hoge respons voor bunker en ruwe olie. Dit verschil kan verklaard worden door verschillen in de soorten en concentraties van agonisten aanwezig in de verschillende oliën, die op hun beurt verklaard kunnen worden door de verschillen in afkomst van de oliën. Experimenten waarbij oliën in combinatie met de standaarden (dat wil zeggen BaP of TCDD) werden getest toonde aan dat deze oliën een antagonistische of additieve werking hebben .

In aanvulling op AhR activatie, gaf de aanwezige literatuur ook aan dat sommige ruwe oliën en zware stook oliën *in vitro* ook (anti)oestrogene en antiandrogene activiteiten kunnen vertonen. Echter een aantal vragen blijft onbeantwoord. Onder andere de vraag of de waargenomen activiteiten alleen gelden voor ruwe- en stook olie en of één type olie meer dan een effect kan induceren (bv. één type olie die zowel oestrogene als androgene activiteiten heeft). Daarom zijn de endocrien verstorende eigenschappen van de oliën getest in **Hoofdstuk 3** en **Hoofdstuk 4.** In **Hoofdstuk 3** is de (anti) oestogeniciteit en de (anti) androgeniciteit bestudeerd door gebruik te maken van een recombinante gist, stabiel getransfecteerd met humaan receptor alpha (ERα), beta (ERβ), of de androgeen receptor (AR), die het gist geoptimaliseerde groen fluorescerend eiwit tot expressie brengt. Geen van de geteste oliën liet significante oestregene effecten in de ERα assay of androgene effecten in de AR assay zien. Alle geteste olien waren echter in staat om oestrogene effecten te induceren in de ERβ assay, sommige zelfs tot 160% van de maximale inductie van de positieve controle, 17β-estradiol (E2). Gebaseerd op de laagste effect concentraties, waren de potenties van de oliën in alle testen tussen de 4 en 7 ordes van grootte lager dan die van de standaarden E2 of testosteron (T). De potenties van de individuele petrochemische agonisten kunnen echter erg hoog zijn, omdat deze maar een heel klein deel uitmaken van de totale massa van de olie (waarop de vermelde potenties gebaseerd zijn). Additieve, antagonistische en een synergistisch effect zijn gemeten in de assay bij een gelijktijdige blootstelling aan vaste concentraties standaard (E2 of T) en een toenemende concentratie van de olie. Deze vormen van combinatie effecten als gevolg van blootstelling aan mengsels kunnen nadelige gevolgen hebben voor de ontwikkelingsfase tijdens de organogenese en reproductie cycli van in het wild levende dieren. Gegeven het feit dat veel effecten op cellulair niveau worden gereguleerd via de ER en AR, zoals cel proliferatie en synthese en secretie van specifieke eiwitten, gaat **Hoofdstuk 3** ook in op de vraag of deze waargenomen effecten receptor gemedieerd zijn. Om deze reden zijn er experimenten gedaan waarbij de gist tegelijkertijd wordt blootgesteld aan de synthetische inhibitoren ICI 182,780 (ERβ assay) of flutamide (AR assay), een vaste concentratie standaard en verschillende concentraties olie. De resultaten suggereerden dat de androgene effecten receptor gemedieerd zijn, terwijl de oestrogene effecten maar gedeeltelijk via de receptor gemedieerd worden. De gist bio-assays die in dit proefschrift gebruikt zijn, zijn snelle, gemakkelijke en robuuste hulpmiddelen om te onderzoeken of complexe oliën de hormoonhuishouding kunnen ontregelen. Echter, de verschillen tussen gistcellen en zoogdiercellen in transporters, co-activatoren, co-repressors en metabolische stress benadrukken de noodzaak om oliën ook in zoogdiercellen assays te testen. Om te kijken of deze oestrogene effecten die zichtbaar zijn in gistcellen ook

te zien zijn in zoogdiercellen, zijn de oliën ook getest in recombinante zoogdiercellijn assays die stabiel getransfecteerd zijn met de humane ERα of ERβ en die het luciferase gen tot expressie brengen (bv., ERα-U2OS-Luc and ERβ-U2OS-Luc assay). Deze experimenten worden bediscussieerd in **Hoofdstuk 4**. In beide assays induceerden alle oliën, behalve twee geraffineerde en één ruwe olie, een oestrogene respons.
De relatieve oestrogene potenties van de oliën waren 6 tot 9 orders van grootte lager dan de potentie van E2. Na het tegelijkertijd blootstellen aan een vaste concentratie van E2 en toenemende concentraties olie, werden mogelijke additieve, antogonistische en synergistische effecten waargenomen. Andere mechanismen dan receptor activatie, zoals receptor fosforylatie en DNA-receptor complex formatie effecten zouden mogelijke verklaringen kunnen zijn voor dit fenomeen. Andere auteurs suggereren dat de *in vitro* synergistische responses zijn gerelateerd aan het aantal Estrogen Responsive Elements (EREs), hun onderlinge afstand en de aard van de promoter. Bovendien is een van de meest potente oliën, een nautische stookolie, ook getest in een E-Screen assay met de oestrogeen afhankelijke humane borst carcinoma cellijn (MCF-7). De olie induceerde cel proliferatie tot wel 70% relatief aan de maximale inductie door E2. Bij 25 mg/L, de laagste concentratie waarbij de olie een effect had, induceerde de olie ook mRNA expressie van het oestrogen afhankelijke eiwit pS2 met een factor twee. Deze resultaten geven dus aan dat behalve eiwit secretie oliën andere effecten kunnen induceren en ze ondersteunen het bestaande bewijs dat olie kan interfereren meat de oestrogene en androgene signaleringsrouten.

De *in* vitro assays die zijn toegepast in **Hoofdstuk 2, 3** en **4** ondersteunen de gevaren identificatie en karakterisatie van deze voor het milieu relevante oliën. Hoewel de olie alomtegenwoordig is in het milieu, wordt de interne blootstelling van een organisme aan deze petroleum koolwaterstoffen vooral bepaald door het potentieel van deze verbindingen om te bioaccumuleren. Het bioaccumulatie potentieel van de petroleum koolwaterstoffen hangt waarschijnlijk af van hun hydrofobiciteit; hoewel er verder weinig bekend is over het lot van specifieke chemicaliën in de oliën die toxische effecten veroorzaken. Na een olieramp zullen binnen 24 uur de meest vluchtige verbindingen, zoals lage kookpunt fracties en mono-aromatische koolwaterstoffen, al verdampt zijn. Andere verbindingen met een hoog kookpunt, zoals PAH's die bekend staan om hun lipofiliteit, zullen in het milieu achterblijven en worden daarom gezien als problematisch. Bovendien verhoogt een groter bioaccumulatie potentieel de kans op toxische effecten en is het dus belangrijk voor de verdere bepaling van de milieurisico's van deze verbindingen. In hoofdstuk 5 wordt de bioaccumulatie van petroleum koolwaterstoffen in de, in het water levende, worm *Lumbriculus variegates* aangetoond. De bio-geaccumuleerde olien werden uit de wormen verkregen en vervolgens werden de extracten gedoseerd in de *in vitro* chemisch geactiveerde fluorescerend reporter gen bio- assay (CAFLUX) om de AhR activerende effecten aan te tonen. Testen van asphaltenic, alifatische, aromatische, en resin olie fracties in de DR-Luc experimenten tonen aan dat gemakkelijk te metaboliseren AhR agonisten aanwezig zijn in

zowel de aromatische als resin fracties van alle oliën. Na 24 uur blootstelling was de AhR activatie vooral in de aromatische fractie te vinden. Verdere fractionering van de aromatische en resin fracties van oliën geven aan dat er veel verschillende chemicaliën zijn die verantwoordelijk zijn voor de AhR-gemedieerde effecten, omdat deze activiteit werd gevonden in fracties met een verschillende hydrofobiciteit, variërend van log *Kow* van 4 tot 9. Dit houdt in dat deze chemicaliën variëren van matig tot super-hydrofoob en dat ze een matig tot extreem hoog bioconcentratie en bioacculumaltie potentieel hebben

De belangrijkste conclusies van dit proefschrift zijn:

(i) Behalve chemicaliën die narcosis kunnen veroorzaken bevatten geraffineerde en ruwe oliën ook chemicaliën die specifieke toxische effecten kunnen veroorzaken

(ii) Niet alleen aromatische- maar ook polaire componenten in olie zijn verantwoordelijk voor de AhR activerende effecten

(iii) De meeste chemicaliën die verantwoordelijk zijn voor het activeren van de receptor zitten in de hoge kookpunt fracties en deze staan bekend om hun hydrofobiciteit

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Curriculum Vitae

Cozmina Maria Vrabie was born on November 9, 1979, in Galati, Romania.
In 1998 she started the five years bachelor study in Biotechnology at Dunarea de Jos University, Galati, Romania. In January 2002, she had the opportunity to join as an Erasmus exchange student, the Biotechnology Master programme at Wageningen University, The Netherlands.
In the same year she was offered a MATRA scholarship that allowed her the opportunity to enroll in the Environmental Sciences Master programme from Wageningen University. Her MSc thesis research was performed under the close supervison of Dr. Merijn Schriks and Prof. Tinka Murk at Toxicology Department, Wageningen University. During this period she studied the agonism and antagonism of xenobiotics on cellular thyroid hormone action.
After completing her MSc studies at the end of 2003, she started working as a student assistant under the supervision of Prof. Tinka Murk. The main activity focused on carrying out *in vitro* research on thyroid hormone-like activities of PBDEs as a part of the EU-FIRE project.

In December 2004 she joined the Fungal Genomics Section, Wageningen University under the supervision of Prof. J. A. van den Berg. During a period of 7 months she carried out research on soil bioremediation using Aspergillus niger.

In Septermber 2005 she started her PhD project at Institute for Risk Assessment Sciences (IRAS), Utrecht University under the supervision of Dr. Chiel Jonker, Dr. Majorie van Duursen and Prof. Martin van den Berg. During her PhD project she worked as well in close collaboration with Prof. Tinka Murk.

Starting February 2010, Cozmina is employed as Regulatory Affairs Manager at NOTOX B.V., The Netherlands.

List of Publications

Cozmina M. Vrabie, Theo L. Sinnige, Albertinka J. Murk, Michiel T.O. Jonker, Effect-directed assessment of the bioaccumulation potential and chemical nature of Ah Receptor agonists in crude and refined oils. Submitted to Environmental Sciences and Technology.

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