eISBN: 978-1-68108-427-5
ISBN: 978-1-68108-428-2

PETROGENIC POLYCYCLIC AROMATIC HYDROCARBONS IN THE AQUATIC ENVIRONMENT:

ANALYSIS, SYNTHESIS, TOXICITY AND ENVIRONMENTAL IMPACT

Editors:

Daniela M. Pampanin

Magne O. Sydnes

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Petrogenic Polycyclic Aromatic Hydrocarbons in the Aquatic Environment: Analysis, Synthesis, Toxicity and Environmental Impact

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eISBN (Online): 978-1-68108-427-5

ISBN (Print): 978-1-68108-428-2

Published by Bentham Science Publishers – Sharjah, UAE.

First published in 2017.

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Denise Fernandes, Anna Marque¶o, Cinta Porte and *Montserrat Sol®*

Emil Lindbª ck and *Magne O. Sydnes*

FOREWORD

Research about polyaromatic hydrocarbons (PAHs) as environmental stressors is a long term topic with multiple aspects. Its importance implies that we will not see any final papers within the area for a long time. As the research unfolds, both extensive global reviews and more limited reviews addressing recent or expected advances are needed. As new investigative methods emerge it is useful to sum up recent findings and the possible directions being opened to us. This is the purpose of this book.

The analysts now refer to the oil fingerprinting field as “petroleomics”, indicating how the field is advancing based on more powerful tools. The analytical advances are not only regarding the parent PAH compounds, but also the metabolized PAHs, known for their high hazardousness. High resolution mass spectrometry will probably soon lead to the detection of a wider range of metabolized and oxidised PAHs too, which will need follow up in different directions.

The analysts ability to synthesize reference PAH metabolites is one of them, and new research challenges will be posed regarding their potential environmental effects. Other logical following steps are revisits of the PAH compounds bioavailability, biodegradability and carcinogenicity. This is the reason these themes are taken up in this book, accompanied by other advances within each of their respective topics. Finally, suggestions are given to how the advances will open for new monitoring methods and strategies leading to one of our important goals: an improved control of a challenged marine environment for which there is an increasingly growing concern.

May this book be an important drop in the ocean that diminishes the PAH part of that concern and help fellow researchers and ourselves to be on top of this complex issue!

Steinar Sanni
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PREFACE

Crude oil and consequently polycyclic aromatic hydrocarbons (PAHs) have been released to the marine environment long time before humans discovered oil through natural oil seeps. Nature has quite efficient systems for cleaning up these releases of contaminants. Since the first human oil excavation started at sea, many oil spills of small and large sizes, have been taking place (chapter 1, 2 and 3). These types of incidents represent an overdose to the natural processes.

The natural defense system for oil degradation is micro organisms, mainly bacteria, which can use the components in oil as an energy source (chapter 7). However, these bacteria preferably use the readily available straight chain hydrocarbons as their first choice of nutrition. This results in a very slow natural removal of larger components such as PAHs, which requires more processing in order to utilize the energy found in the molecules. With great abundance of easily accessible energy sources available, *e.g.* alkanes, which is the case in an oil spill, PAHs will accumulate in the environment. The micro organisms will only start breaking down these compounds in order to get energy once other energy sources are used up. In the meantime PAHs, which have been pointed out as the most toxic organic compounds found in oil, will impact marine life.

Research has shown that PAHs cause severe effects on fish at all life stages, with the most severe effects found on fish embryos. The book will convey an up to date overview of the current state of knowledge regarding the negative health effects of PAHs on marine life (chapter 1 and 4). Most of the health problems related to exposure to PAHs are caused by the oxidized metabolites that organisms generate *in vivo* in order to excrete the unwanted compounds (chapter 5). The metabolites are much more reactive and therefore also more toxic than their mother compounds (chapter 4 and 5). These secondary compounds are prone to react with DNA and proteins to form adducts.

Central in research directed towards understanding the mode of action for PAH metabolites *in vivo* has been the preparation of synthetic material (chapter 6). This material has been utilized as standards for analysis and starting point for studying further metabolism *in vivo*. Moreover, due to their presence in the marine environment and their genotoxicity, there is a standing requirement for oil and gas operators to monitor their concentration and influence on marine life through environmental monitoring studies (*e.g.* the Water Column monitoring) (chapter 2).

Although a lot is known about the influence of PAHs on the environment, and the marine environment in particular, there are still many unresolved questions that are awaiting answers. It is our intention that this book will give a solid basis facilitating the pursue of these unanswered questions.

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CHAPTER 1

Introduction to Petrogenic Polycyclic Aromatic Hydrocarbons (PAHs) in the Aquatic Environment

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Abstract: This chapter serves as an introduction to the book. It contains key points and background information that will be followed up and expanded in the coming chapters. Polycyclic aromatic hydrocarbons (PAHs) are a constituent of crude oil and PAHs from petroleum are called petrogenic PAHs. This book focuses on petrogenic PAHs and research results related to them, including sources of contamination in the aquatic environment, analytical methods utilized in order to detect and quantify their presence in various matrixes, biological effects of PAHs and their metabolites, and microbial degradation with special focus on the situation in the Arctic. The tracking of sources of petrogenic contamination is of significance and it is a focus of this book. Both current knowledge and future challenges related to petrogenic PAHs are introduced and discussed.

Keywords: Biological effects, Biota, Contamination, Crude oil, PAHs.

PETROGENIC PAHS

Polycyclic aromatic hydrocarbons (PAHs) are a constituent of crude oil and PAHs from petroleum are called petrogenic PAHs. These groups of compounds have been of high concern due to their toxic effect and in particular their carcinogenic potential [1]. PAHs are known for causing adverse effects in aquatic organisms, however, their toxicity is not directly due to the parent compounds, but predomi-

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nantly due to the oxidation products generated *in vivo* [2, 3]. The oxidation products are formed during the process of making the compounds more water soluble so that they can be more easily excreted. There has been a great research effort in order to unravel the harm caused by PAHs on biota. Hylland reviewed the ecotoxicology knowledge related to PAHs in the marine ecosystem, underlining the link between these compounds and their adverse effects on biota and recognizing the importance of the determination of adverse effects using biological variables (*i.e.* biomarkers) [4].

Chapter 2 will give an overview of the possible sources of contamination.

It is important to provide information about the analytical methods utilized to identify the source of contamination (see Chapter 3) and the methods used for their quantification in biota.

Significant improvements in analytical chemistry methods are increasing the possibility to analyse a large fraction of PAH compounds found in oil in a single analysis. Descriptions of these methods, especially new approaches using mass spectrometry, and how they are and can be used in environmental investigations are reported in Chapter 3.

More than 600 aromatic hydrocarbons have been listed, from the monocycle benzene (molecular weight (MW) = 78) to the nine ring compounds (MW up to 478). They are classified according to their physical and chemical properties, *i.e.* the temperature of compound formation and the origin. They can be classified in: 1) natural, *i.e.* biogenic or diagenetic origin; 2) pyrogenic, *i.e.* originated from pyrolysis substrates; 3) petrogenic, *i.e.* originated from petroleum sources. Characterisation of PAHs has been reviewed and physical and chemical properties of the most common PAH contaminants (*e.g.* MW, aqueous solubility, vapor pressure, the octanol-water partition coefficient, boiling point) are available on-line.

The characterization and quantification of PAHs in the sediment compartment have received growing attention since the 1970s (*i.e.* lakes, rivers, estuaries and seas). Most studies during this time reported a predominance of pyrogenic PAHs *versus* petrogenic PAHs, except in specific cases of oil spills or oil related activities. Petrogenic PAHs in general can result in more bioavailability since they tend to bind strongly to sediment particles.

The identification of PAH type is essential for evaluating the risk to biota. The ratio between alkylated PAHs and the parent compound is commonly used to distinguish between PAH types [5 - 7]. In particular, petrogenic PAH composition is dominated by alkyl constituents.

Petrogenic PAHs primarily consists of 2- and 3-ring compounds and alkyltated forms. The alkyltated forms can make up to 90% of the PAHs found in crude oil. It is important to note that alkylated PAHs have higher toxicity than other forms of PAHs.

The concern over the toxicity of PAHs resulted in U.S. EPA (Environmental Protection Agency) establishing the narcosis model for protecting the benthic community [8]. These guidelines require the measurement of the so-called 34 PAHs in sediment sample (*i.e.* 18 parent PAHs and 16 alkyl PAH derivatives) to evaluate the impact of PAH contamination on benthic species [9].

Knowledge and regulations have been improved since the introduction of the 16 EPA PAH concept in the 1970s and more research is focusing on toxicity, environmental and chemical analysis of other polycyclic aromatic compounds, *e.g.* alkylate PAHs, amino-PAHs, cyno-PAHs [10 - 12].

BIOACCUMULATION OF PAHS IN AQUATIC ORGANISMS

Petrogenic PAHs are bioavailable compounds in the aquatic environment and their presence in biota represents a problem. The estimation of the total PAH load entering the aquatic environment is clearly quite difficult and the value of 0.5 million tons/year suggested in the 1980s is now at least one order of magnitude greater.

Most studies related to the measurement of PAH concentration in aquatic organisms are linked to the risk they pose on human health [13 - 17]. Therefore, the management of aquatic resources have been traditionally based only on the quantification of PAHs in biota. PAHs are included in the often referred to as the Hygiene Package from the European regulation, which reports the limits for

CHAPTER 2

The Presence of Petrogenic PAHs in the Aquatic Environment, a Focus on Monitoring Studies

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Abstract: Due to their toxic properties and their carcinogenic potential, petrogenic PAHs are routinely monitored in environmental surveys and following accidental events such as oil spills, boat accidents, and accidental discharges. Measurements of PAH concentrations in water and sediment can give an estimation of their abundance, but this does not reveal the real environmental risk. Bioaccumulation in biota and further biological effects of these compounds on various organisms needs to be evaluated to establish the risk posed to the aquatic environment by their presence. In this chapter, monitoring activities of petrogenic PAHs in the aquatic environment are described, including specific cases as examples. Due to the increased knowledge and technological improvements in recent years, new monitoring strategies are also proposed.

Keywords: Bioaccumulation, Biomarkers, Biomonitoring, Invertebrates, Monitoring, PAHs, Sources of contamination, Vertebrates.

INTRODUCTION

Monitoring of the aquatic environment is extremely important for providing concrete information for the preservation of ecosystems against the adverse effect of anthropogenic sources of contamination. Great attention is focused nowadays on the cocktail of chemicals present in the aquatic environment and their potential

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detrimental influence [1]. Within the cocktail of contaminants, the presence of polycyclic aromatic hydrocarbon (PAH) compounds are of particular concern due to their proven carcinogenic properties [2, 3]. Some refer to PAHs as PBT substances: Persistent, Bioaccumulative and Toxic substances. Sensitive and solid source of contamination trackers are still under development. This is particularly relevant for legal issues and chronic contaminations.

Due to the large number of publications related to techniques and approaches for monitoring the presence of PAHs in the aquatic environment [4], this book chapter will focus on giving an overview of the most recent successfully applied strategies in sediment, water and biota. In particular, the chapter will highlight new promising methodologies for tracking PAH contamination sources in biota that concurrently provide information about the real effect of PAH contamination on the aquatic ecosystem and about the source of contamination.

Causes of the Presence of PAHs in the Aquatic Environment

Petrogenic PAHs are present in the marine environment in significant concentrations [5]. They are naturally present in crude oil and coal. In coastal areas, they enter the water primarily from sewage, runoff from roads, smelter industries and oil spills while the presence of PAHs offshore is mostly related to oil seeps, oil spills and produced water discharge from oil and gas installations [3] (Fig. **1**).

Evaluation of PAH sources and their effects has been reported since the ‘80s, as shown for example in the National Research Council (US) Committee on pyrene and selected analogues [6]. Examples of sources of PAHs in the aquatic environment are reported herein and a selection of relevant references is proposed.

Natural Occurrence

PAHs are naturally found in aquatic plants, in bottom sediments, in fresh and marine waters, and in emissions from volcanoes and forest fires. The natural occurrence of PAHs in the aquatic environment does not represent an environmental risk *per se*. A good example is the study of Olivella *et al.* [7]; the authors reported the evaluation of exceptional leakage of ashes from a forest fire into riverine waters in Catalonia (Spain) in 1994. Even if the studied areas were clearly affected by extensive forest fires (and consequently exceptional emission of PAHs), levels of PAHs in sampled drinking water were always below the limit established by the European Community. This confirms that naturally occurring PAHs are most likely not harmful to the aquatic environment or to human health.

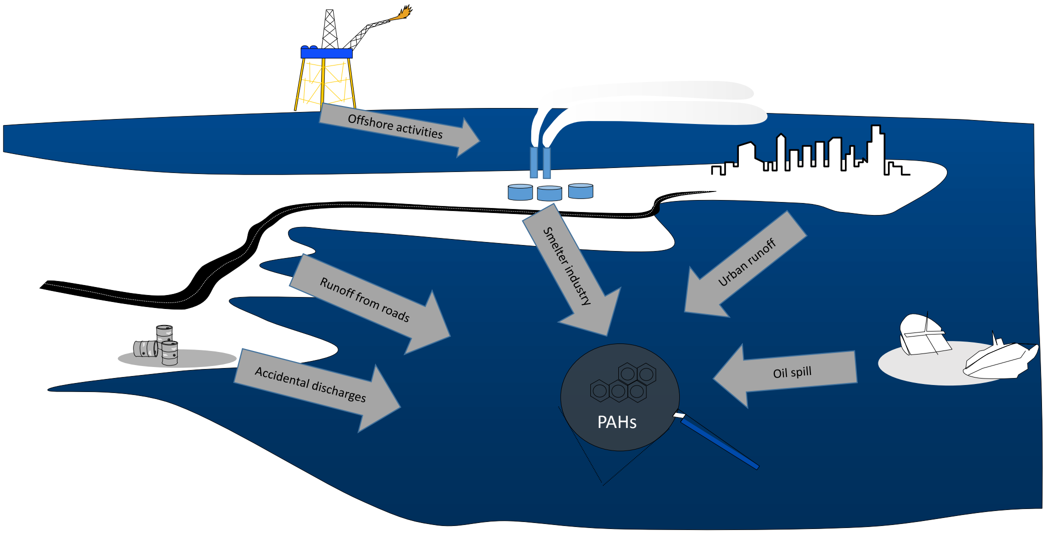

Fig. (1). Anthropogenic sources of PAH contamination in the Sea (figure courtesy of J. Beyer).

Natural processes are also in place to preserve a functional aquatic environment (*i.e.* biodegradation processes, see Chapter 7 of this book and [8]). Nevertheless, the knowledge gained studying the natural presence of PAH represents a good base for the establishment of monitoring tools [9, 10]. Developments in molecular and analytical chemistry have been improving the understanding of the microbial metabolic networks, providing valuable tools to both verify and assess the presence of PAHs [11].

It is important to note that bioremediation, which exploits the natural microbial degradation of organic compounds, is also considered the most cost-effective and sustainable cleaning technology related to PAHs [12].

Oil Exploration and Production

Discharges from oil and gas exploration and production have been subjected to

CHAPTER 3

Oil Spill Fingerprinting – Identification of Crude Oil Source of Contamination

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Abstract: Source identification of oil with its thousands of compounds has always been a great challenge for analytical chemists. With the significant improvements in analytical instrumentation and work-up techniques this challenge has started to become possible to overcome. Lately, the development of new mass spectrometry techniques and the increased sensitivity of the instruments have made it possible to analyze a big fraction of compounds found in oil in one single analysis. The analytical field conducting oil fingerprinting is now commonly referred to as petroleomics. The main focus of this chapter is directed towards how analyses are used in environmental investigations at present and how the technology can be used in the future for oil spill forensics.

Keywords: Analytical chemistry, Crude oil, Mass spectrometry (MS), Oil fingerprinting, Petroleomics, Polycyclic aromatic hydrocarbons (PAHs).

INTRODUCTION

Crude oil enters the environment through both natural processes and accidental spills often caused by human error [1]. Natural oil seeps are found unevenly distributed around the world and crude oil has been leaking from its reservoirs for as long as oil has existed [2]. Initially it was through oil seeps on land where oil naturally had gathered on the surface that originally lead to the discovery of oil.

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The ecosystem around oil seeps, both on land and in the ocean, is adjusted to the conditions caused by slow release of hydrocarbons. In fact, organisms living in such areas, predominantly bacteria, have methods for detoxifying the various compounds found in crude oil. For many of these bacteria the oil becomes an energy source [2]. Bacteria degrades the lighter fractions much faster than the heavier compounds leaving behind the latter [3].

Oil spills caused by human activities generally have larger consequences for the environment then natural oil seeps, due to the large volume of oil entering a location simultaneously. Such spills are caused by blow-outs, leakage during development and production of an oil field, accidents with oil tankers during loading and transportation, and leakage from fuel tanks. For example, two large incidents that recently occurred is the *Exxon Valdez* spill in Alaska in 1989 and the *Deep Water Horizon* blow-out in the Gulf of Mexico in 2010 [4]. Even though it is more than 25 years ago since the *Exxon Valdez* accident took place in Prince Williams Sound, Alaska, there is still research published describing the long term effects of this spill, particularly on fish populations [4].

The two mentioned incidents were very large oil spills, which rarely take place. However, smaller oil spills are taking place quite frequently. Data from the Republic of Korea covering a 19 year period (1990-2008) showed that there were 354 oil spills larger than 50,000 L taking place over that period of time in waters of the coast of the country [5]. This averages out to more than 18 oil spills per year in that area. So oil spills are more common than one might think. Small oil spills are taking place around on the seven seas several times per day (*e.g.* small scale spills take place on almost daily bases in the oceans of the Republic of Korea [5]). Many of these smaller events are not reported and when they are discovered it is difficult to point out the source of the oil contamination. Nevertheless, small oil spills in a sensitive environment, or at a sensitive developmental stage for an organism for example, can have large impact for that species at the site of the spill.

The development in analytical instrumentation over the last 10-15 years has now brought chemists in a position where it is possible to do source identification of crude oil [6]. By such means, it is possible to identify the oil's fingerprint and use that in order to find out where the oil originated from.

Crude Oil

Crude oil is a complex mixture of alkenes (paraffin's), cycloalkanes (naphthenes), aromatics, and asphaltics [7]. The distribution between the different chemical groups varies from oil to oil, and the distribution between the various compounds categorized in the same group also varies significantly between oils from different sources. The variations is so large that petroleum from different origins has its own unique combination and concentration of compounds, *i.e.* a unique fingerprint, which is possible to identify due to modern analytical techniques. Table **1** summarizes the average distribution between the major compound groups found in crude oil and highlights the large composition range that is found in crude oil.

Table 1. Composition by weight of compound groups found in crude oil [7].

Hydrocarbon	Average	Range
Alkanes (paraffins)	30%	15-60%
Cycloalkanes (naphthenes)	49%	30-60%
Aromatics	15%	3-30%
Asphaltics	6%	remainder

The aromatic fraction of crude oil can vary from only 3% to 30% (Table **1**). Benzene, toluene, and xylene (*o*-xylene, *m*-xylene, and *p*-xylene) make up the majority of the quantity of aromatic compounds found in crude oil. Polycyclic aromatic hydrocarbons (PAHs) only accounts for a minute amount of the aromatic fraction [1, 8]. However, PAHs are the compounds of most concern due to their persistence in the environment and their toxicological properties (*i.e.* carcinogenicity) [9 - 11]. Similarly to the large variation of aromatic compounds in crude oil, there is also a large difference in the concentration of the various PAHs found in oil [1]. The concentration of the different PAHs strongly influence the toxicity of an oil since the toxicity of the various PAHs is greatly different, or more precisely the toxicity of the metabolites formed *in vivo* [1, 9, 11]. In general, the higher the concentration of PAHs with more than 3 rings is in the oil the more toxic the PAH fraction of the oil will be after metabolization [11].

CHAPTER 4

Carcinogenicity of Petrogenic PAHs

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Abstract: The carcinogenicity associated with PAH contamination in the aquatic environment has been a topic of prime importance since neoplasia was described in fish living in multi-sources contaminated areas, 50 years ago. Since then, a whole array of studies were conducted in order to better characterize the fate of PAHs, their bioavailability for biota and their effects in term of neoplastic lesions. Genotoxicity assessment is at the heart of the matter, as benzo[*a*]pyrene, the leader of PAHs, is described as ubiquitous genotoxic and carcinogenic compound, acting in particular by the occurrence of reactive diol metabolite that forms DNA adducts. The causal relationship between exposure to PAHs and occurrence of neoplasia in organisms involves to explore the biological plausibility of the association. Biomarkers of genotoxicity/mutagenicity are a central part of tools that measure the biological plausibility. DNA adducts and DNA strand breaks are lesions that can be advantageously used in sentinel organisms.

Keywords: DNA, DNA adducts, Genotoxicity, Neoplasia, Polycyclic aromatic hydrocarbons (PAHs).

INTRODUCTION

Many polycyclic aromatic hydrocarbons (PAHs) are known to be toxic for humans and other organisms, with well described acute and chronic effects [1]. In general, it is admitted that one-, two- or three-ring aromatic compounds have predominately acute effects on living organisms, while higher molecular weight PAHs are mainly responsible for chronical health impacts, among them carcinogenicity in relation (or not) with genotoxic/mutagenic potential [2]. The

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exploration of a structure-activity relationship suggests that carcinogenicity in PAH family is promoted by a structural arrangement in a so-called fjord (or bay) region that requires molecules of at least four aromatic rings [1]. This distinction is far from being absolute, especially because interactions between PAHs, and PAHs and other pollutants, are of wide variety and are contributing to highly variable qualitative and quantitative global toxic effects [3]. Dioxin- like responses in interaction with the aryl hydrocarbon receptor (AhR) are also observed in different biological models exposed to PAH mixtures or isolated compounds. Results are still inconsistent and mechanisms are still relatively unknown [4, 5]. Many PAHs and derivatives, notably alkylated compounds that are predominant among PAHs in crude oils, are supposed to be endocrine disrupters and, at least partly, exert carcinogenic effects through non genotoxic mechanisms including tumor promotion [6]. Retene (1-methyl-7-isopropyl phenanthrene), an alkyl PAH used in chemical fingerprinting of crude oils, has for example been responsible for the embryotoxicity and other dioxin like adverse effects in marine medaka, probably by a toxic mechanism different from TCDD and its parent phenanthrene [7].

The determination of the mutagenic potential associated with single chemical or mixtures can be considered as a crucial endpoint in highlighting the causal relation between exposure and cancer development (even though numerous carcinogens are not mutagens). In this way, some work realized on *in vitro* and *in vivo* models of toxicology reflect the mutagenic/carcinogenic potential of certain petrogenic PAHs. Seven complex mixtures of petroleum derived PAHs and numerous isolated PAHs (among them phenanthrene, anthracene, methylanthrancène, dimethylbenzo[*c*]phenanthrene) have proven to be mutagen by the *in vitro* reverse mutation bacterial test (Ames test), in presence of S9 fraction (used to mimic the mammalian metabolic conditions) [8]. In a similar assay, the mutagenic potential of benz[*a*]anthracene, benzo[*a*]pyrene (BaP) and 25 methylated metabolites was directly associated to the chemical formula and more precisely to the positioning of methyl groups around the aromatic structure [9]. An activation of the cellular response to DNA damage including the phosphorylation of the P53 protein is observed when rat liver stem-like cells are exposed to methylated BaPs [10]. DNA adducts, as marker of genotoxicity, have been observed too. Non genotoxic mechanisms of carcinogenicity are potentially associated to 1-MeBaP and 3-MeBaP as agonists for AhR. Interestingly, mutagenic potential of PAHs in mixture can be different than the sum of the effect caused by each agent alone. By measuring mutations of the hprt gene in Hepa-1c1c7 cells (a mouse hepatoma cell line), Huang *et al.* showed that fluoranthene is able to reduce significantly the mutagenic impact of BaP when cells are co-exposed [11]. This effect is not dependent of the AhR nor the CYP1A1. This *in vitro* study highlights once again the complexity of global effects of pollutant mixtures.

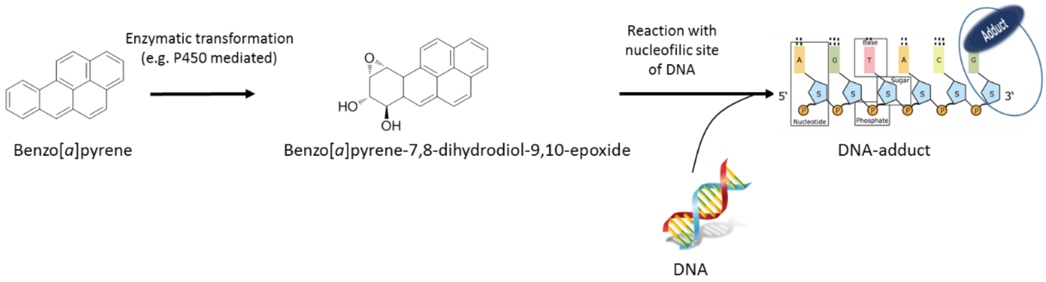

Fig. (1). Example of a PAH metabolisation that generates an electrophilic form that can directly react with a nucleophilic site of DNA.

On *in vivo* experimental models, some well characterized PAHs proved to be carcinogens, mutagens and teratogens. Some of them are responsible for respiratory and cardiovascular diseases too. On human, epidemiologic studies tend to corroborate these experimental data. BaP is considered the leader of carcinogenic PAHs. It is also the most studied to date, being used as a PAH model compound, notably as regards the metabolism pathways. In numerous toxicologic model organisms, PAHs are not direct carcinogens, and necessitate the enzymatic biotransformation by cytochrome P450 monooxygenases in order to form *in situ* highly reactive metabolites. Phase I enzymes, such as cytochrome P450s, catalyse the monooxygenation of PAHs leading to phenols and epoxides. As ever mentioned, the best documented example is BaP, which metabolisation generates the well-known benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (BPDE), an electrophilic diol epoxide that can directly react with nucleophilic sites of DNA, RNA, lipids and proteins (Fig. **1**). These interactions with DNA and the formation

CHAPTER 5

PAH Metabolites in Fish and Invertebrates: Analysis and Endocrine Disruptive Potential

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Abstract: This chapter describes the metabolism of PAHs to oxidized and conjugated metabolites by both fish and invertebrates. So far, a wide range of metabolites have been described and a number of studies have alerted for the potential of phenol and quinone derivatives, as well as other reactive intermediates, to exert toxicity in early life stages of fish and reproductive disorders in adults. The endocrine disruptive properties have been mainly related to action at the receptor level, due to structural similarities of PAH and their metabolites with natural hormones, and also to interaction with key steroidogenic enzymes, which are present in vertebrate and invertebrate groups. The fast development of new analytical techniques, based on the use of high resolution mass spectrometry, will lead in the near future to the detection of a wider range of oxidized PAHs in abiotic matrices (*i.e.* water, sediment) and hopefully in biota, bringing along new research challenges.

Keywords: Analysis, Endocrine alteration, Metabolism, Oxidized PAHs.

PAH METABOLITE FORMATION IN AQUATIC SPECIES

In the last decade, the analysis of PAH metabolites has gained importance in front of the more traditional analysis of parent compounds. It was wisely pointed out that the analysis of only parent compounds in fish may underestimate exposure to PAHs [1]. Since then, PAH metabolites have been screened for in muscle, gut and gills from different fish species [2]. Although bile remains the

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most frequently analyzed matrix, and the presence of PAH metabolites in bile has been successfully used as a biomarker of PAH exposure in field studies [3]. PAH metabolites have also been successfully determined in the urine of crabs [4, 5]. For other invertebrate species, the absence of an easily accessible biofluid makes the tissue analysis necessary in order to determine PAH exposure. Often the whole animal [6, 7], or specific tissues, such as muscle or visceral mass, are analyzed [8 - 10]. Recently, Layshock *et al.* detected the presence of PAHs substituted with keto- or quinone functional groups [11], namely, 9-fluorenone, 9,10- anthraquinone, benzofluorenone, and 7,12-benz[*a*]anthracenequinone in mussels, alerting that levels of oxygenated PAHs (OPAHs) could be similar or even exceed those of parental PAHs. This is one of the first studies reporting the presence of PAH metabolites in molluscs, as usually, OPAH are generated in fish and other aquatic organisms possessing well-developed enzymatic detoxification systems that efficiently convert PAHs to epoxide and hydroxylated derivatives, among others, during phase I metabolism (*e.g.* cytochrome P450 isoenzymes). These derivatives are further converted by phase II enzymes into highly water-soluble conjugates, such as glucuronides or sulphates that are readily excreted [7, 8].

Two of the main commonly phase I metabolites formed by vertebrates, and some invertebrates, (*e.g.* 1-hydroxypyrene and 9-hydroxyphenanthrene) share a structural resemblance to the natural hormone estradiol and this characteristic confers them estrogenic properties in humans and fish [12]. In addition, the conjugated forms of these phase I metabolites could also be deconjugated in the aquatic environment by microbial processes [13]. Thus, in the last years, concern has been raised about the disruptive potential of not only PAHs but also their metabolites over the endocrine reproductive regulation in fish and invertebrates. In this context, the scope of this review is to report on the different metabolic capacities of fish and invertebrates exposed to either parental or substituted PAHs and/or some of their main metabolites. Biotransformation of PAHs will be placed in the context of metabolites identified in vertebrates and invertebrates. It is thanks to recent advances in analytical technologies that progress on the identification of PAH metabolite formation in different aquatic species has been made. Tissue distribution of PAH metabolites will be described for fish and invertebrates collected from different field environments or exposed to crude oil and/or specific PAHs in the laboratory. In addition, already reported evidences of endocrine reproductive disturbances in aquatic species of both groups exposed to either parental PAHs and/or their respective metabolites will be considered.

PAH Metabolites in Fish

Fish have long been considered to possess a well developed enzymatic system able to biotransform PAHs into more polar metabolites and in this way eliminate them from their body compartments. Nonetheless, during these biotransformation reactions and due to some particular metabolite formation, toxicity and other endocrine disrupting events may arise [3]. The metabolic profile originated may also differ due to several factors even within fish. Species, age, physiological status, tissue distribution, way of exposure, pre-exposure conditions may be some factors that account for these differences and they have often been explored (see Table **1** for a summary).

Table 1. PAH metabolites identified in vertebrate and invertebrate tissues using different liquid- or gas-chromatography analytical methods.

Metabolites analyzed	Matrix	Sample treatment	Analytical method	Reference
1-, 2-Hydroxynaphthalene.	Fish bile	SPE + NH_2 cleanup + derivatization (BSTFA)	GC-MS (SIM)	[14]
	Fish bile	SPE + derivatization (BSTFA)	GC-MS (SIM)	[15]
1-Hydroxynaphthalene	Octopus	MeOH/ethyl acetate (1:1)	SPE-LC-FD	[7]
(1*R*,2*R*)-1,2-dihydronaphthalene-1,2-Diol.	Fish bile	Dilution ($MeOH:H_2O$; 1:1)	F and SFS	[16]
1-Naphtoic acid.	Mussels	Soxhlet (DCM) (A) + saponification (B)	HPLC-UV (A) and GC-MS (B)	[17]
1,4-Naphthoquinone; 1-naphthaldehyde; 1,2-acenaphthenequinone; 1,8-naphthalic anhydride; naphthene-5,12-dione.	Fish (muscle and guts and gills)	ASE + filtration + silica gel column.	GC-MS (SIM)	[2]
2-Hydroxybiphenyl.	Fish bile	SPE + NH_2 cleanup + derivatization (BSTFA)	GC-MS (SIM)	[14]

CHAPTER 6

Synthesis of Environmental Relevant Metabolites

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Abstract: Polycyclic aromatic hydrocarbons are metabolized *in vivo* resulting in the formation a range of oxidized products – metabolites. The metabolites generated are more water soluable and therefore easier to excrete from the system, but these compounds are also more toxic for the organism. A range of PAH metabolites have been synthesized and used in order to study their toxicity and further faith *in vivo*. Different synthetic strategies have been used in order to prepare the metabolites. Herein, the synthetic strategies utilized for the formation of environmental relevant metabolites of naphthalene, acenaphthene, fluorine, phenanthrene, and chrysene, the PAHs found with the highest concentration in crude oil, are described in detail.

Keywords: Acenaphthene, Chrysene, Diols, Fluorine, Naphthalene, Oxidation, Phenanthrene, Phenols.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are enzymatically metabolized *in vivo* in organisms in order to make these compounds more water soluble. A range of oxidation products are formed in these processes with a large variation of products and product distribution varying from organism to organism due to species specific metabolism [1, 2]. In order to evaluate the toxicity of the generated metabolites and study their further faith *in vivo* it is necessary to obtain the metabolites in pure form.

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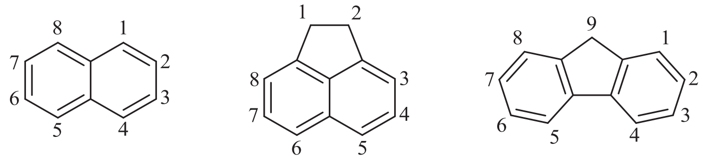
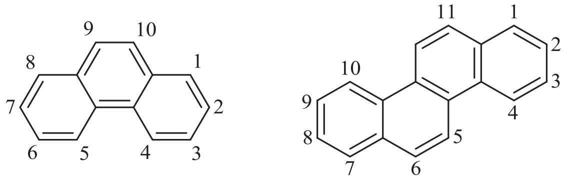
During metabolism of PAHs a range of phenols, diols, and epoxides are formed. In the case of phenols different regioisomers are generated, and diols and epoxides are formed as different regioisomers with specific stereochemistry. For the chiral metabolites the toxicity of the compounds is very dependent on their stereochemistry. For biological studies it is therefore desirable to utilize metabolites with high enantiomerical excess (*ee*) in order to assure that an observed biological effect can be linked to a specific enantiomer.

The best method to generate sufficient quantities of these specific metabolites required for biological studies is through total synthesis. There are two main strategies to consider when designing a total synthesis of diols and epoxides. Either an asymmetric synthesis, or a racemic synthesis. The latter strategy requires a final separation in order to isolate the two enantiomers formed, and by such means obtain the enantiomerically pure compounds.



Naphthalene (**1**) Acenaphthene (**2**) Fluorene (**3**)



Phenanthrene (**4**) Chrysene (**5**)

Fig. (1). The structure of naphthalene (**1**), acenaphthene (**2**), fluorine (**3**), phenanthrene (**4**), and chrysene (**5**) with indication of how the carbons are numbered.

Many of the relevant metabolites generated *in vivo* from the EPA 16 PAHs [3] have been prepared synthetically. These syntheses have been presented in a large amount of publications over the last 50-60 years. In preparing this chapter, it was necessary to decide which parent PAHs should be focus. In crude oil, there is a great variation in concentration of the various PAHs. However, there are a few PAHs that are generally present in higher concentrations than other PAHs found in crude oil. It was therefore decided to focus on the few PAHs that generally make up the majority of the concentration of PAHs found in crude oil. Therefore, the focus herein will be on the reported synthesis of metabolites that can be formed *in vivo* by oxidation of naphthalene (**1**), acenaphthene (**2**), fluorine (**3**), phenanthrene (**4**), and chrysene (**5**) (Fig. **1**).

Naphthols

Formation of Naphthols by Hydroxylation of Halonaphthalenes

In the recent years, metal catalyzed, in particular copper catalyzed, direct hydroxylation of halonaphthalenes has emerged as an attractive methodology for the synthesis of naphthols. As outlined in Scheme **1** and Scheme **2**, several examples exist where both 1-naphthol (**6**) and 2-naphthol (**7**) have been obtained by copper catalyzed direct hydroxylation of the corresponding halonaphthalenes in the presence of various hydroxide salts. For instance, You and co-workers concluded that 1-naphthol (**6**) could be obtained in very good yield when 1-iodonaphthalene (**8**) was coupled with the hydroxide ion of potassium hydroxide employing copper iodide as a precatalyst in the presence of 1,10-phenanthroline as a ligand (Scheme **1**, Conditions A) [4]. Likewise, Wang and colleagues also used copper iodide as a precatalyst for the direct hydroxylation of 2-iodonaphthalene (**9**) with potassium hydroxide as the hydroxyl source and tetrabutylammonium bromide (TBAB) as phase-transfer catalyst [5] using triethanolamine as ligand (Scheme **1**, Conditions B) [6].

In contrast to the examples presented in Scheme **1**, Conditions A-B, the Chae research-group utilized copper(II) as the copper source for the copper catalyzed direct hydroxylation of both 1- (**8**) and 2-iodonaphthalene (**9**) [7]. In fact, the use of copper(II) hydroxide in combination with glycolic acid as ligand afforded 1-naphthol (**6**) and 2-naphthol (**7**) in 98 and 77% yield, respectively (Scheme **1**, Conditions C). Another efficient copper source for the direct hydroxylation of 1- (**8**) and 2-iodonaphthalene (**9**) is $Cu_3(btc)_2$ (btc = 1,3,5-benzenetricarboxylate (Scheme **1**, Conditions D) [8].

CHAPTER 7

Microbial Degradation in the Aquatic Environment

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Abstract: Considering their recalcitrance and toxicity, understanding how PAHs are removed from the marine environment is essential in order to maintain healthy and functional ecosystems. This chapter discusses the major advances in knowledge in the field of polycyclic aromatic hydrocarbon (PAH) biodegradation regarding key players, degradation mechanisms, genetic background, environmental factors influencing biodegradation and the role of interaction among microbial community members. Microbial degradation, or biodegradation, is the natural mechanism by which PAHs are channeled back into the marine carbon cycle and transformed into harmless material. The key players in this process are specialized bacteria, which developed strategies to utilize PAH as carbon and energy source. The rate of biodegradation is one of the major concerns in terms of bioremediation efforts. Factors influencing the rate at which PAHs are converted include temperature, oxygen concentration, availability of nutrients, type and bioavailability of PAHs, and the level of adaptation of the local microbial community.

Keywords: Biodegradation, Marine hydrocarbon degrading bacteria, PAHs.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are among those constituents of crude oil, which tend to persist, are highly toxic, accumulate in higher-level organisms and some of them are also well-known carcinogens [1]. Due to these characteristics, PAHs represent high risk to the marine environment. Understanding the processes contributing to their elimination is essential for better

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mitigation of ecosystem damages. The overall fate of PAHs in seawater includes photo-oxidation, uptake and transformation by higher-level organisms, sedimentation (following adsorption to particulate matter) and microbial biodegradation. Each of these processes has a unique outcome and contribution to contaminant fate. Nevertheless, it is only microbial degradation which can completely eliminate PAHs from the environment, by transforming them into harmless products (*e.g.* carbon-dioxide and water) and cell material [2, 3]. Owing to their ability to biodegrade toxic compounds and thereby recycle carbon and energy from pollutants, microbial communities play an essential role in maintaining functional marine ecosystems. Firstly, by reducing the amount of harmful chemicals they directly decrease the environmental impact of anthropogenic pollution. Secondly, the microbial cell material produced from consuming contaminants represents a readily available carbon source for other higher-level members of the marine food web [4 - 7].

Biodegradation in general can be defined as a process in which microorganisms decompose organic molecules into simpler chemicals and thereby, ideally, transforming harmful compounds into non-harmful ones [3]. In this context, complete biodegradation occurs when compounds are transformed into water and carbon dioxide through catabolic (energy producing) processes and into cell material through anabolic (carbon assimilating) processes. The former is often referred to as mineralization. Incomplete or partial biodegradation involves the formation of intermediary metabolites, which can be released back into the environment. Very little research has been done regarding the fate of such intermediary metabolites excreted from microorganisms, despite the fact that some of these compounds are known to be more toxic than the parent molecules [8, 9].

From an environmental risk perspective, both the extent and the speed (kinetics) of biodegradation is important to consider. They depend on the type and abundance of microorganisms involved, the bioavailability of contaminants and various environmental factors (*e.g.* nutrient availability and temperature). The aim of this chapter is to outline current knowledge regarding each of these subtopics. First it will introduce the broader diversity of microorganisms (both eukaryotic and prokaryotic) involved in marine PAH biodegradation, and then the fundamental mechanisms will be discussed with focus on aerob heterotrophic bacterial processes. Finally, environmental factors and the role of interaction within the microbial loop will be discussed.

By definition, microorganisms are single celled, and invisible to the naked eye. Both prokaryotic domains, Bacteria and Archaea, fulfil this requirement, while among Eukaryotes, only Algae, Fungi and Protozoans harbour microbes. Most members of this microbial world have been to a varying extent studied in connection with PAH removal from marine environments. Aerob heterotrophic bacteria are by far the most broadly studied group. While the focus of this chapter will be the aerob heterotroph PAH degrading bacteria, current knowledge regarding the other microbial groups will also be described.

Hydrocarbon Degrading Microorganisms

Crude oil, one of the major sources of marine PAH pollution, is a naturally occurring highly complex mixture of organic components. Its major hydrocarbon groups include alkanes, resins, and asphaltenes besides PAHs. Crude oil has been released into the environment spontaneously throughout historical times. According to estimates by the National Research Council (USA) in 2003, nearly 50% of all the oil entering the marine environment originated from natural oil seeps, amounting to 700 million liters each year globally [10]. Though estimates vary widely, natural seepage is certainly a significant source of crude oil in the oceans [11, 12]. This continuous presence of hydrocarbons had a crucial role in the evolution of specialized microorganisms, which were able to develop mechanisms for utilizing hydrocarbons, mainly alkanes and PAHs, as growth substrates. Already in the 1940s, Claude Zobell published two reviews summarizing rich evidence for marine (and also terrestrial) microorganisms being able to grow on unusual materials, such as asphalt, rubber and wax [13, 14]. Since then, petroleum microbiology as a research area developed and the diversity of hydrocarbon degrading microorganisms has continuously been emerging. It was quickly understood that crude oil in the sea is degraded according to a pattern of sequential depletion defined by the biodegradability of hydrocarbon groups. In terms of biodegradability, PAHs are approximately in the middle of the scale where short, straight-chained alkanes are the most easily, and large asphaltenes

SUBJECT INDEX

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