Chapter from the book *Hydrocarbon*

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1. Introduction

Crude oil is a complex mixture of hydrocarbons containing more than 17000 compounds [1]. Among the constituents of crude oil there is a group of substances called polycyclic aromatic hydrocarbons (PAHs). PAHs are aromatic compounds containing from two to eight conjugated ring systems. They can have a range of substituents such as alkyl, nitro, and amino groups in their structure [2]. Nitrogen, sulfur, and oxygen atoms can also be incorporated into their ring system [2,3]. The precursors for PAHs found in crude oil are natural products, such as steroids, that have been chemically converted to aromatic hydrocarbons over time [4].

The PAHs that are present in the marine environment in relevant concentrations are divided into two groups depending on their origin, namely pyrogenic and petrogenic [5]. Pyrogenic PAHs are formed by incomplete combustion of organic material while the petrogenic PAHs are present in oil and some oil products [4,6,7]. In general the pyrogenic PAHs are composed of larger ring systems then the petrogenic PAHs. Sources for pyrogenic PAHs are forest fires [6,7,8], incomplete combustion of fossil fuels [6,7,8], and tobacco smoke [6,7]. A range of PAHs are naturally present in crude oil [4,9,10] and coal [10,11] and these compounds are referred to as petrogenic PAHs. In the costal zones PAHs enters the water primarily from sewage, runoff from roads [12], the smelter industry [13,14,15] and oil spills [16,17], while offshore PAHs chiefly enter the water through oil seeps [18], oil spills [16], and produced water discharge from offshore oil installations [19].
2. Oil as a source of polycyclic aromatic hydrocarbons to the aquatic environment

Hydrocarbons in the form of crude oil, and therefore also PAHs, have and are entering the environment naturally through oil seeps, which is oil leaking naturally from oil reservoirs. Oil seeps are found scattered all over the globe with a higher concentration in certain regions of the world [18]. Numerous times the presence of a natural oil seep has resulted in the discovery of oil reservoirs that are large enough for commercial oil production [20], however, the presence of an oil seep does not guarantee the discovery of a production worthy oil reservoir [21]. Oil seeps vary in size with macroseeps resulting in visible oil slicks on the water surface, when the oil seep is situated on the seafloor, and microseeps that are invisible at the surface [22]. Estimates for the world-wide seepage rate vary between 0.02-2.0 x 10^6 tons per year with the most realistic estimate being 0.2 x 10^6 tons per year [23]. The presence of natural oil seeps also results in local presence of hydrocarbon-eating microorganisms [24,25], a fact that gives these regions an advantage in the case of an accidental oil spill [24,26]. For example resent research showed that the presence of oil eating microorganisms due to natural oil seeps in the Gulf of Mexico in addition to favorable water currents resulted in a quicker degradation of oil in the region after the Deepwater Horizon accident than otherwise would be expected [26,27]. As expected, it is the lighter fractions, viz. short chain alkanes, of the oil that are first degraded by microorganisms [28]. The easily accessible energy source, short chain alkanes, results in an explosion like increase in the number of the oil degrading microbes. After some time these microorganisms also start degrading the more complex molecules such as long chain alkanes and aromatic compounds like PAHs [29]. A range of PAH degrading bugs has been found naturally in the environment [30-34]. These microorganisms have been isolated, sequenced and studied extensively in the laboratory, and their mechanism of degrading PAHs is fairly well understood [29].

For monitoring purposes The US Environmental Protection Agency has made a list of 16 unsubstituted PAHs that are on a priority pollutant list [35]. These PAHs are usually referred to as the EPA 16 PAHs (Figure 1) and are the PAHs most commonly analyzed for. The concentration of these 16 PAHs and other PAHs, and the ratios between the various compounds differ from oil to oil [36,37]. This fact is quite clearly presented in the work of Kerr et al. where they have analyzed the concentration of the prioritized 16 PAHs in 48 crude oils from around the globe (North America, South America, Africa, and Asia) [36]. Their results are summarized in Table 1 and show an enormous variation of the content of the various PAHs in crude oil from different sites. Crude oil from the North Sea is reported to have a PAH concentration of 0.83% [38] while for example crude oil that leaked out of Exxon Valdez (referred to as Exxon Valdez crude oil hereafter) had a PAH content of 1.47% [39].

Naphthalene, one of the 16 EPA PAHs, is present in the highest concentration in crude oil, nevertheless this quantity does not give the full details of the naphthalene content of the oil. In fact, if the content of methylated naphthalene, a normal constituents of crude oil, is also
included in the analysis the total naphthalene concentration is much higher as illustrated in Table 2. A similar situation is also found for phenanthrene [38,39] and chrysene [38], while for the less abundant PAHs in crude oil excluding the alkylated derivatives of the parent compounds does not alter the total quantity significantly. In fact the alkylated PAHs found in crude oil have been reported to be more toxic then their unsubstituted congeners [41-43].

<table>
<thead>
<tr>
<th>Crude oil</th>
<th>PAH</th>
<th>Minimum mg/kg oil</th>
<th>Maximum mg/kg oil</th>
<th>Mean mg/kg oil</th>
<th>North Sea [38]</th>
<th>Goliat [40]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>1.2</td>
<td>3700</td>
<td>427</td>
<td>1169</td>
<td>1030</td>
<td></td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>0</td>
<td>58</td>
<td>11.1</td>
<td>18</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorene</td>
<td>1.4</td>
<td>380</td>
<td>70.34</td>
<td>265</td>
<td>75</td>
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<td>17</td>
<td>4.3</td>
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<td>Phenanthrene</td>
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<td>400</td>
<td>146</td>
<td>238</td>
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<td>10</td>
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<tr>
<td>Pyrene</td>
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<td>9.2</td>
<td>-</td>
<td>20</td>
<td>*</td>
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<tr>
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<td>0</td>
<td>16</td>
<td>2.88</td>
<td>11</td>
<td>Na^c</td>
<td></td>
</tr>
<tr>
<td>Chrysene</td>
<td>4</td>
<td>120</td>
<td>30.36</td>
<td>26</td>
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<td></td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>0</td>
<td>14</td>
<td>4.08</td>
<td>4.2</td>
<td>Na^c</td>
<td></td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>0</td>
<td>1.3</td>
<td>0.07</td>
<td>Nd^a</td>
<td>Na^c</td>
<td></td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>0</td>
<td>7.7</td>
<td>1.5</td>
<td>1.3</td>
<td>Na^c</td>
<td></td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>0</td>
<td>7.7</td>
<td>1.25</td>
<td>Nd^a</td>
<td>Na^c</td>
<td></td>
</tr>
<tr>
<td>Benzo[g,h,j]perylen</td>
<td>0</td>
<td>1.7</td>
<td>0.08</td>
<td>1</td>
<td>Na^c</td>
<td></td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>0</td>
<td>1.7</td>
<td>0.08</td>
<td>Nd^a</td>
<td>Na^c</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Minimum, maximum, and mean PAH content in 48 different crude oils [36], and PAH content in two North European crude oils, North Sea crude oil [38], Goliat crude oil [40] (^Nd = not detected; bGoliat is situated in the Barents Sea; Na = not analyzed for).

<table>
<thead>
<tr>
<th>Crude oil</th>
<th>North Sea [38]</th>
<th>Goliat [41]</th>
<th>Exxon Valdez crude oil [39]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH</td>
<td>mg/kg oil</td>
<td>mg/kg oil</td>
<td>mg/kg oil</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>1169</td>
<td>1030</td>
<td>720</td>
</tr>
<tr>
<td>N-Methylnaphthalene</td>
<td>2108</td>
<td>2700</td>
<td>1330</td>
</tr>
<tr>
<td>2-Methylnaphthalene</td>
<td>2204</td>
<td>4200</td>
<td>1020</td>
</tr>
<tr>
<td>3-Methylnaphthalene</td>
<td>1172</td>
<td>2800</td>
<td>Na^a</td>
</tr>
</tbody>
</table>

Table 2. Concentration of naphthalene and the three regioisomers of methylnaphthalene in crude oil from three different locations (^Na = not analyzed).

PAHs represent the group of compounds in oil that has received the greatest attention due to their carcinogenic and mutagenic properties [38,44]. More precisely, intermediates are formed that are far more toxic than the mother compounds during cellular detoxification of the PAHs in vivo [45]. Due to the toxicity of the PAH metabolites the oil industry in some
areas of the world (e.g. North Sea, Mediterranean Sea, Australian Northwest Shelf, Gulf of Mexico) are required to monitor their discharges to the aquatic environment [46-49]. In the North Sea this is taken care of through the Water Column Monitoring programs [48]. For monitoring purposes the 16 EPA compounds has been chosen as the most important PAHs to analyze for.

Figure 1. Chemical structure of the EPA selected 16 PAHs.

Unfortunately, accidental oil spills take place from time to time, most recently the Deepwater Horizon accident in the Mexican Gulf in 2010 (April 20th). This incident resulted in the release of 779 million liters of crude oil to the Gulf [50]. The Exxon Valdez oil spill in Prince William Sound, Alaska, USA in 1989 (March 24th), which took place after the oil tanker ran ashore, resulted in the release of 42 million liters of crude oil [16]. In European waters the Erika accident in 1999 (December 12th) released 18000 tons of crude oil into French coastal waters and in Spain Prestige spilt 60000 tons of heavy fuel oil into the waters outside of Galicia in 2002 (November 13th) [17]. These are unfortunately only a few examples of accidental release of large quantities of oil to the aquatic environment. The environmental consequences of the Exxon Valdez spill is the most studied oil spill ever [51,52], however, the influence of the Deepwater Horizon oil release will probably be just as well studied, or even more studied [53].

3. Produced water as a source of polycyclic aromatic hydrocarbons to the aquatic environment

Oil production offshore (Figure 2) (and onshore) also results in the production of large volumes of water, so called produced water (PW), in addition to crude oil. PAHs contained in
PW are receiving much attention due to their potential for causing adverse effects in the marine environment [54,55]. PW generally consists of a mixture of: 1) formation water contained naturally in the reservoir; 2) injected water used for the recovery of oil; and 3) treatment chemicals added during production [19]. Data from offshore oil production platforms in the North Sea have showed that the major aromatic compounds in PW are BTEX (benzene, toluene, ethylbenzene and xylene) (97%), 2- and 3-ring PAHs (3%) named NPD (naphthalenes, phenanthrenes and dibenzothiophenes) and larger PAHs (<0.2%) [19,56,57].

The average concentrations of NDPs and the remaining 14 PAHs from the EPA 16 list are shown in Table 3. With regard to the NPD group, the lowest molecular weight substance, naphthalene represents the largest fraction (92%). Even PAHs with more than 3-rings constitute less than 0.2% of the aromatic content of PW, with the concentrations of the individual compounds decreasing with the number of rings in their structure, by about one order of magnitude for each additional ring.

<table>
<thead>
<tr>
<th>16 PAHs from the EPA list</th>
<th>Oil installation</th>
<th>Gas installation</th>
<th>Unspecified installation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>145</td>
<td>115</td>
<td>108</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>13.6</td>
<td>20.9</td>
<td>10.7</td>
</tr>
<tr>
<td>Fluorene</td>
<td>8.3</td>
<td>13.1</td>
<td>6.7</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>2</td>
<td>50.1</td>
<td>1.78</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>0.86</td>
<td>12.6</td>
<td>2.35</td>
</tr>
<tr>
<td>Fluoranthenes</td>
<td>0.26</td>
<td>35.4</td>
<td>0.29</td>
</tr>
<tr>
<td>Anthracene</td>
<td>3.74</td>
<td>110</td>
<td>1.17</td>
</tr>
<tr>
<td>Pyrene</td>
<td>0.63</td>
<td>8</td>
<td>0.47</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>0.52</td>
<td>-</td>
<td>0.022</td>
</tr>
<tr>
<td>Chrysene</td>
<td>0.84</td>
<td>1</td>
<td>0.52</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>0.23</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Benzo[b]fluoranthenes</td>
<td>0.028</td>
<td>-</td>
<td>0.031</td>
</tr>
<tr>
<td>Benzo[k]fluoranthenes</td>
<td>0.007</td>
<td>-</td>
<td>0.007</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>0.005</td>
<td>-</td>
<td>0.005</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylenes</td>
<td>0.029</td>
<td>-</td>
<td>0.019</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>0.005</td>
<td>-</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Table 3. Mean concentrations of aromatic compounds in produced water (expressed as µg/L) (data from OLF, UKOOA and OGP companies, [58]).

PAHs in PW are present in both dissolved and dispersed (oil droplets) forms. Existing oil/water separators, such as hydrocyclones, are efficient in removing droplets, but not dissolved hydrocarbons from PW. Therefore, much of the PAHs discharged in to the marine environment are dissolved low molecular weight compounds. Nevertheless, since the treatment procedures are not 100% effective, discharged PW still contains some dispersed oil (droplet size from 1 to 10 µm) [59]. Concentration of total PAHs in PW typically range from 0.040 to 3 mg/L (Table 4) and consist primarily of the most water-soluble congeners.
such as naphthalene, phenanthrene and their alkylated homologues (2- and 3-ring PAHs). The abundance of these alkyl substituted PAHs is also higher than for the parent compounds (the non-alkylated homologues). Higher molecular weight PAHs (up to 6-ring) are in fact rarely detected in properly treated PW [60,61]. The ratio between alkylated-PAHs and the corresponding parent compounds is therefore used to confirm the nature of the pollution source in field studies [54,62].

Due to their lipophilic properties, PAHs are mainly associated with dispersed oil droplets [59,63]. In fact, it has been documented that up to 10% of the total PAHs in PW from a platform on the Northwest Shelf of Australia were in the dissolved fraction being formed mainly by alkynaphthalenes and alkylphenanthrenes [64]. Moreover, these droplets also contained almost all the dibenzothiophenes, fluoranthenes/pyrenes and chrysenes present in the PW.

**Figure 2.** The sources of PAHs entering the marine environment offshore are predominantly natural oil seeps, oil spills from boats or platforms, and produced water discharge from oil and gas producing installations such as the one shown. PAHs in produced water and oil seeps represent a chronic release to the marine environment.

As a chronic source of PAH contamination in the marine environment, PW is also a source of concern with respect to possible long term impact on the environment [65]. Estimates of the PW discharge volumes on the Norwegian shelf predict an increase until 2010-2014, reaching a maximum of about 200 million L/year [66]. Therefore, this offshore discharge is currently under periodical monitoring [48,67].
<table>
<thead>
<tr>
<th>Compound</th>
<th>Gulf of Mexico</th>
<th>North Sea</th>
<th>Scotia Shelf</th>
<th>Grands Bank (Canada)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>5.3-90.2</td>
<td>237-394</td>
<td>1512</td>
<td>131</td>
</tr>
<tr>
<td>C1-Naphthalenes</td>
<td>4.2-73.2</td>
<td>123-354</td>
<td>499</td>
<td>186</td>
</tr>
<tr>
<td>C2-Naphthalenes</td>
<td>4.4-88.2</td>
<td>26.1-260</td>
<td>92</td>
<td>163</td>
</tr>
<tr>
<td>C3-Naphthalenes</td>
<td>2.8-82.6</td>
<td>19.3-81.3</td>
<td>17</td>
<td>97.2</td>
</tr>
<tr>
<td>C4-Naphthalenes</td>
<td>1.0-52.4</td>
<td>1.1-75.7</td>
<td>3.0</td>
<td>54.1</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>ND-1.1</td>
<td>ND</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>ND-0.1</td>
<td>0.37-4.1</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Biphenyl</td>
<td>0.36-10.6</td>
<td>12.1-51.7</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Fluorene</td>
<td>0.06-2.8</td>
<td>2.6-21.7</td>
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<td>16.5</td>
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<tr>
<td>C1-Fluorene</td>
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<td>1.1-27.3</td>
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<td>ND</td>
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<td>C4-Phenanthrene</td>
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<td>Pyrene</td>
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<td>C1-Fluoranthene/Pyrenes</td>
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<td>ND</td>
<td>9.1</td>
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<td>0.60</td>
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<td>0.06-4.4</td>
<td>ND</td>
<td>6.3</td>
</tr>
<tr>
<td>C2-Chrysenes</td>
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<tr>
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<td>4.2</td>
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<tr>
<td>Benzo[b]fluoranthene</td>
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<td>Benzo[k]fluoranthene</td>
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<td>Pyrelene</td>
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<td>ND</td>
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<tr>
<td>Indeno[1,2,3-cd]pyrenec</td>
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<td>0.022-0.23</td>
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<td>ND</td>
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<tr>
<td>Dibenz[a,h]anthracene</td>
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<td>0.012-0.10</td>
<td>ND</td>
<td>0.21</td>
</tr>
<tr>
<td>Benzo[g,h,j]perylene</td>
<td>ND-0.03</td>
<td>0.01-0.28</td>
<td>ND</td>
<td>0.17</td>
</tr>
<tr>
<td>Total PAHs</td>
<td>40-600</td>
<td>419-1559</td>
<td>2148</td>
<td>845</td>
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</tbody>
</table>

**Table 4.** Concentrations of individual PAHs or alky congener groups in produced water from various areas (expressed as µg/L) (ND = not detected) (from [61]).
4. Environmental monitoring of polycyclic aromatic hydrocarbons

There are various environmental monitoring methods which may be performed in order to assess risks of PAH contamination for organisms and to classify the environmental quality of ecosystems. Five approaches are hereby reported: 1) chemical monitoring: exposure assessment by measuring levels of a selected set of compounds in abiotic environmental compartments; 2) bioaccumulation monitoring: exposure assessment by measuring PAH levels in biota or determining the critical dose at a critical site (bioaccumulation); 3) biological effect monitoring: exposure and effect assessment by determining the early adverse alterations that are partly or fully reversible (biomarkers); 4) health monitoring: effect assessment by examining the occurrence of irreversible diseases or tissue damage in organisms; 5) ecosystem monitoring: assessment of the integrity of an ecosystem by making an inventory of, for instance, species composition, density and diversity [68-70]. All these methods are currently in use to monitor the aquatic environment contamination from PAH compounds.

Since the occurrence and abundance of PAHs in aquatic environments represent a risk to aquatic organisms and ultimately to humans (through fish and shellfish consumption), there is a constant need for their determination and quantification around the world [71]. The monitoring of PAH presence in the aquatic environment is therefore a world-wide activity. Since some of these compounds are well known carcinogens and mutagens [44,72], this contaminant class has been generally regarded as high priority for environmental pollution monitoring. In fact, the European Union included these pollutants in the list of priority hazardous substances for surface waters in the Water Framework Directive 2000/60/EC [73]. Moreover, for PAH content in biota several guidelines exist: the commission regulation (EC 2005) which stipulates a maximum concentration of 10 µg/kg (w/w) of benzo[a]pyrene in edible molluscs (this regulation also limits the benzo[a]pyrene concentration in other alimentary products), the OSPAR Commission which has developed eco-toxicological assessment criteria (i.e., concentrations levels above which concern is indicated) for different PAHs in fish and mussels [74], and the Oregon Health Division which has derived risk-based criteria for PAHs [75] assessing the risk in terms of benzo[a]pyrene equivalents.

Many studies have been carried out to determine the PAH distribution in marine organisms in different geographical areas using different approaches. Different authors have reported the presence of PAHs in waters, marine organisms, and sediments using chemical and biological markers [76-78]. Current monitoring techniques employed to determine environmental quality include the chemical analyses of sediment and water samples for determining the concentration of PAH parent compounds. PAHs are sparingly soluble in water and are difficult to detect although they show a much greater association with sediments. In fact, due to their hydrophobic character, these compounds rapidly tend to become associated with particles and end up in the sediments which act as a sink for them [71,79,80]. High concentrations of pyrogenic PAH mixtures in sediment samples have been found in several freshwater, estuarine and marine regions with heavy vessel traffic or at locations with PAH-containing effluents from industrial areas [81]. Finally, chemical
analyses of these materials are laborious and costly due to the lengthy extraction methodology needed. Since monitoring of a large number of PAHs is expensive, time consuming and analytically demanding, a selection of compounds could be monitored to give an indication of the overall contamination.

Standard procedure for determining PAHs in the aquatic environment have a long history that covers at least three decades. A few examples are reported here (see also Table 5). One of the first official procedures was published in 1985 by HMSO (Her Majesty’s Stationery Office) and provided the analysis of six PAHs in water samples [82]. In 1986, the EPA published EPA procedure 8310 for determining PAHs by HPLC [83]. In 2007, the EPA published the EPA procedure 8270D for determination of 16 PAHs applying gas chromatography coupled with mass spectrometry (GC-MS) [84]. To close, the standard method for PAH determination in water recommended by the International Organization for Standardization (ISO), ISO 17993:2002, is based on liquid-liquid extraction and final determination by HPLC [85].

<table>
<thead>
<tr>
<th>Method name</th>
<th>Application</th>
<th>Sample preparation</th>
<th>Determination technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA-610</td>
<td>PAHs in municipal and industrial wastewaters</td>
<td>About 1L of water samples is extracted with dichloromethane, the extract is then dried and concentrated to a final volume of less than 10 mL.</td>
<td>HPLC or GC (with a packed column)</td>
</tr>
<tr>
<td>EPA-8310</td>
<td>Groundwater and wastewater</td>
<td>EPA 3500 series methods (version 3, 2000)</td>
<td>HPLC</td>
</tr>
<tr>
<td>EPA-8270D</td>
<td>Aqueous samples</td>
<td>EPA 3500 series methods (version 3, 2000)</td>
<td>GC-MS with use of high resolution capillary columns and deuterated internal standard</td>
</tr>
<tr>
<td>ISO 17993:2002</td>
<td>Water quality (determination of 15 PAHs)</td>
<td>Water samples collected in brown glass bottles are stabilized by adding sodium thiosulfate. 1 L of sample is extracted using hexane. The extract is dried with sodium sulfate and then enriched by removal of hexane by rotary evaporation</td>
<td>HPLC and fluorescence detector</td>
</tr>
</tbody>
</table>

Table 5. Standard procedures for determining of PAHs in aqueous samples (modified from [86]).

Modern analyses have at their disposal a wide spectrum of tools, specialized analytical equipment and standard procedures that are capable of providing high quality results even if intercalibration studies and commonly accepted SOP are needed [86].
Chemical fingerprinting of a PAH mixture can lead to the identification of the contamination source, for example an oil spill accident [87]. PAH compounds in a petrogenic exposure often contain one or more methyl-ethyl- or butyl-(and sometimes higher alkyl-) groups on one or more of the aromatic carbons [88]. For example, the ratio between alkyl-PAH compounds and the corresponding parent compounds is commonly used to confirm the nature of the pollution source in field studies from PW discharges (i.e. the abundance of alkyl substituted PAHs is higher than for the parent compounds (the non-alkylated homologues)). It has been shown that mussels caged down-stream of PW discharges from oil platforms accumulate higher concentrations of alkynaphthalenes, alkylphenanthrenes and alkyl dibenzothiophenes, than their respective parent compounds [62]. In a recent study, the ratio of alkylated over non-alkylated PAHs indicated a diffuse petrogenic contamination in the Ekofisk area (up to 2000 m from PW fallout) [55].

PAHs co-occur in various amounts and the composition of the mixture differs depending upon the source from which they are derived and their subsequent degradation. For example a recent study clearly demonstrated the correlation between PAH contamination in river water and the nearby activity of textile factories [89]. A widespread PAH contamination in the San Francisco Bay has been reported after a 10 year monitoring of water, sediment and biota samples [90]. Since the process of industrialization and urbanization is growing rapidly in South America, the potential increase in PAH contamination is under evaluation through a number of surveys in the coastal areas, collecting information about the PAH concentration in water, sediment and biota [91-93]. Of course, oil spills are well-known examples of PAH contamination in the aquatic environment. Unfortunately, many cases of studies are reported in the literature from different parts of the world, such as the Gulf of Mexico in [94], the Mediterranean Sea [95], the Spanish coasts [96], and the Philippines islands [97].

PAH contamination is also constantly under the attention of different countries in relation to oil and gas explorations [98]. For example the Water Column Monitoring program financed by Oljedindustriens Landsforbund (OLF), has provided information about PAH contamination from platform discharges in the North Sea since 2001, almost yearly based [48]. This monitoring program is a good example of integration of chemical monitoring methods with biological effect monitoring approaches in PAH monitoring.

Passive sampling devices, such as semipermeable membrane devices (SPMDs) and polar organic chemical integrative samplers (POCIS) also provide a useful contribution to the monitoring of PAH contaminants in the aquatic environment [99-101]. The principle of the passive sampling technique is the placement of a device in the environment for a fixed period of time, where it is left unattended to accumulate contaminants by diffusive and/or sorptive processes. The main advantages of using passive sampling devices over traditional discreet spot water samples are: 1) concentrations are time-integrative during exposure, compensating for fluctuations in discharges; 2) lower detection limits are normally achievable as a larger sample has been taken and; 3) only the freely dissolved and thus more readily bioavailable fraction is measured. Furthermore, there is a reduction in the need for the use of animals in scientific experiments. However, relating passive sampling device
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accumulations to the overall ecological relevance of contaminants is complicated and effects can only be inferred.

Nevertheless, the chemical approach does not provide information about PAH bioavailability, and the toxic potential and the risk posed by the potentially much more toxic daughter compounds produced by many organisms as a result of metabolism and biotransformation of the parent chemicals is not taken into account [102]. Therefore, a biological effect monitoring approach using living organisms for PAH environmental monitoring has been developed during the last couple of decades. Metabolites arising from biotransformation processes may be concentrated in body fluids, tissues or excreta and the analysis of such biological compartments provides an opportunity to detect and measure exposure of organisms to bioavailable contaminants [103]. On the other hand, PAHs are known to induce toxic effects at the individual level [104,105], and integration of chemical analyses with biomarker responses in organisms has been recommended for monitoring of PAH contamination, in particular in oil related activities (e.g. offshore exploitation activities) [48]. The feasibility of using tissue concentrations of PAH compounds in marine species as a marker of environmental contamination depends on the relative rates of uptake, biotransformation and excretion of the organism. Invertebrate filter feeders, such as *Mytilus* spp., are highly efficient accumulators and bioconcentrators of PAHs and therefore commonly used [54,106-108]. Total amounts of the EPA 16 PAHs between 10 µg/kg and 20 µg/kg, have been found in mussels caged in the vicinity of Norwegian platforms [48,54,109]. Fish and other invertebrates, rapidly biotransform PAHs and their presence in tissues is low and no representative of the overall contamination. PAHs related to offshore operational discharges in fact are generally not found in muscle of wild specimens of fish collected in regions with oil and gas activity [110].

Some biomarkers are widely used as sensitive and early warning signals of exposure to PAHs. For example, many studies indicated that PAH compounds were detectable several kilometers away from North Sea oil production platforms using *in vitro* bioassays and biomarkers [111]. Currently the induction of ethoxyresorufin-O-deethylase (EROD) activity, the production of bile metabolites and the formation of DNA adducts have shown the greatest potential for identifying level of exposure to PAHs following contamination of the aquatic environment [87,112-114]. The historically commonly used marker is the induction of Cytochrome P450 1A (CYP1A) in fish measured by the catalyzed O-deethylation of ethoxyresorufin in hepatic microsomes [104,115-118]. The induction of CYP1A in fish following exposure to certain classes of organic contaminants has been the basis of the use of the cytochrome P450 system as a biomarker in pollution monitoring since the 70’s [104]. Many studies reported the used of this biomarker in various monitoring surveys since the ‘80s [104,119,120]. In many cases, EROD activities or CYP1A protein levels were correlated with environmental levels of CYP1A-inducing chemicals such as PAHs. Nevertheless, a linear dose–response relationship cannot always be found between the PAH concentration and the CYP1A content and/or activity in the natural environment, where a mixture of both inducers and inhibitors of CYP1A may act simultaneously [121]. Moreover, other factors (e.g. temperature, season or sexual hormones) can also modulate the responsiveness of the
CYP1A system in fish [122]. Since PAH compounds are absorbed via gills and may be metabolized before reaching the liver, hepatic EROD activity may not be the only and most sensitive organ to reflect the presence of CYP1A inducing agents (such as PAHs) in water. Therefore, a sensitive method to determine EROD activity in gill filaments has also been developed [123].

A very efficient tool to assess PAH exposure in fish is the determination of PAH metabolites in fish bile. They can be measured using several analytical methods from the simple and fast fluorescence assay (fixed fluorescence detection or synchronous fluorescence spectrometry) to the HPLC with fluorescence detection (HPLC-F) after deconjugation, extraction and derivatization of the bile samples, to the extremely sensitive and advanced LC-MS/MS and GC-MS/MS methods. These methods are very different both in regard to their analytical performances towards different PAH metabolites as well as in technical demands and monitoring strategies. A recent review reported the state of the art for the different methods for determining metabolites of PAH pollutants in fish bile [87]. This approach has also been developed for crustaceans. Metabolites in crabs urine have been analyzed to monitor environmental contamination from PAH with success [103,124]. Regarding DNA adduct analysis as a biomarker of exposure to PAHs, a description of methods is reported in the section DNA adducts from polycyclic aromatic hydrocarbons.

5. Polycyclic aromatic hydrocarbon metabolites

The strongly hydrophobic PAHs accumulate in fatty tissue such as liver, where they penetrate the cells by means of passive diffusion. Inside the hepatocytes, PAHs are oxidized and hence made more water-soluble and more reactive by enzymes with arylic hydrocarbon hydroxylase activity to form epoxides and diols according to the general route outlined in Figure 3 [125]. CYP1A is the most important and best described arylic hydrocarbon hydroxylase enzyme participating in the first step (Phase I) of xenobiotic detoxification [126,127]. CYP1A-derived metabolites generally have a high affinity to nucleic acids and proteins, which may result in adduct formation and possible impaired function of these biomolecules.

![Figure 3. General outline of the metabolic degradation of PAHs.](image)
The vast majority of Phase I-derived PAH metabolites are passed on to Phase II (most notably glutathione transferase) and Phase III (most notably the transmembrane ATP-binding cassette exporter proteins) to eventually become excreted with the bile fluid [126]. In this context, the presence of PAH metabolites in the bile of fish is a highly regarded biomarker of recent PAH exposure (exposure that has taken place within a few days prior to sampling and analysis) (vide supra) [37,87].

The various PAHs form a range of metabolites in vivo. Most of the studies mapping these metabolites have been performed on humans, rats, mice, and hamsters [128]. However, some studies have been conducted on fish and/or fish cells. The results from these studies show that the point of oxidation varies from species to species for the same PAH due to the presence of various cytochrome P450 (CYP) isoforms [128]. For fish it is predominantly CYP1A’s role in PAH metabolism that has been studied, however, for humans, in particular, other isoforms of CYP have also been well investigated. For a general discussion regarding the various CYP families found in fish, see Uno et al. [129]. Figure 4 summarizes the data reported for oxidation site for a range of PAHs, phenanthrene [130,131], chrysene [131,132], pyrene [130,133], benz[a]pyrene [131,134], benzo[c]phenanthrene [135,136], and dibenzo[a,l]pyrene [135-137], based on in vitro tests with fish CYP1A. Most of the PAHs shown in Figure 4 have predominantly one major site where oxidation takes place, thus, indicating a high regioselectivity in the enzymatic oxidation by CYP.

![Figure 4. Point of oxidation with distribution intervals.](image-url)

The absolute stereochemistry of PAH metabolites, derived from metabolism of PAHs in the liver, influences the toxicity of the metabolite [138,139]. In particular the diols with R,R-configuration and the R,S-diol-S,R-epoxides show high carcinogenic activity [139]. As shown in Figure 4 for chrysene the predominant diol formed is the 1,2-diol (formed in 58%), with 3,4- and 5,6-diol being formed in 24% and >1%, respectively (structures for the diols are shown in Figure 5) [132]. The data for chrysene depicted in Figure 4 were based on in vitro tests utilizing liver microsomes from brown bullhead, however, these findings were later confirmed by Jonsson et al. in in vivo tests with Atlantic cod [140]. Close to 90% of the chrysene 1,2-diol is formed with the R,R-configuration (structure shown in Figure 5) and
slightly more than 10% is formed with the S,S-configuration [132]. For the 3,4-diol 97% is formed with the R,R-configuration (structure shown in Figure 5) [132], thus indicating that in the fish brown bullhead predominantly the most toxic metabolites are formed. The diols derived from chrysene are only considered to have weak carcinogenic activity [141-143], however, 1,2-dihydroxy-1,2-dihydrochrysene is the starting point for the biosynthesis of the most carcinogenic chrysene metabolite 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrochrysene [144].

Figure 5. Chemical structure of chrysene metabolites.

Figure 6 and 7 summarizes the structure of the metabolites formed by in vivo oxidation of benzo[c]phenanthrene [145], and benzo[a]pyrene, respectively [146,147]. The two major metabolites formed from benzo[c]phenanthrene are compounds 1 and 2. Biological testing revealed that metabolite 3 from benzo[a]pyrene was not carcinogenic while compound 4 was carcinogenic (Figure 7) [146,147]. The carcinogenic metabolite was also the major compounds formed biosynthetically.

Figure 6. Chemical structure of benzo[c]phenanthrene metabolites.

Figure 7. Chemical structure of 7,8-diol-9,10-epoxide-benzo[a]pyrene.

In vivo oxidation in rodents of dibenzo[a,l]pyrene, which is considered as the most potent carcinogenic PAHs, results in a range of metabolites as outlined in Figure 8 [148]. The study showed that predominantly the (-)-11R,12R-enantiomer was formed and that the genotoxic events mainly took place by stereoselective activation of that enantiomer.

The PAHs bay region diol epoxide has been singled out as the cause for this group of compounds carcinogenic activity [149-151]. The diol epoxides have been found to react with cellular macromolecules of paramount importance, namely DNA and proteins [152-154].
Miller proposed in 1970 that PAH metabolites are electrophiles that react with nucleophiles \textit{in vivo}, thus delivering their biological effect [155]. Later it has been proposed that mechanistically the diol epoxides are electrophiles that alkylate the purine bases in DNA via an S\(\text{N}1\)-like epoxide ring opening process [156,157].

![Chemical structure of bibenzo[a,l]pyrene metabolites formed \textit{in vivo} [148].](image)

In the worst scenario, however, adducts are formed between a PAH metabolite and an oncogene in the genomic DNA, hence switching off the cell’s ability to enter into programmed cell death (apoptosis) and provoking the onset of cancerogenesis. The best described example is DNA adduct formation between the pro-apoptotic p53 gene and benzo[a]pyrene-diol-epoxide after metabolic transformation of benzo[a]pyrene by the combined actions of CYP1A and epoxide hydrolase, another Phase I enzyme (this is also the mechanism by which cigarette smokers develop lung cancer). The presence of DNA adducts in liver tissue is, unlike CYP1A-induction or accumulation of PAH metabolites in bile, the result of cumulative exposure over weeks or even months [158,159]. Hence, hepatocytic CYP1A induction, accumulation of PAH metabolites in bile and elevated liver DNA adducts represent a chain of events that, although partly separated in time, are tightly interrelated from a mechanistic point of view. These three biomarkers have, therefore, received much attention and represent valuable biomarkers of PAH-exposure and effect in fish [159-162]. Analogous with several studies on mammals, responses of these core biomarkers of oil exposure have been associated with genotoxic effects such as liver neoplasia [163].
6. DNA adducts from polycyclic aromatic hydrocarbons

The toxicity of PAHs is a continuous subject of intense investigation. The carcinogenic potential of PAHs was recognized as early as 1933 by Cook et al. [164], who isolated a cancer-producing hydrocarbon from coal tar. Many PAHs act as potent carcinogens and/or mutagens via DNA adduct formation. Aquatic vertebrates such as fish are capable of metabolizing PAHs (vide supra), producing reactive intermediates, occasionally with the formation of hydrophobic DNA adducts as an end result.

Metabolic activation of PAHs to reactive intermediates is a prominent mode of their toxic action. Xue and Warshawsky described the principal metabolic pathways that yield reactive PAH intermediates [165]. Two pathways in particular produce electrophiles that may covalently bind to DNA (forming a DNA adduct): 1) electrophilic diol-epoxides from sequential PAH oxidation by cytochrome P450 (CYP) enzymes, hydrolysis of the resulting arene oxides by microsomal epoxide hydrolase, and a second CYP-catalyzed oxidation; 2) one electron oxidation of PAHs by CYP peroxidase yields the radical anion.

There are 18 potential sites for adduct formation in DNA. The specificity of reactions at different sites depends on the reactive species, nucleophilicity of the DNA site and steric factors. The spectra of DNA adducts resulting from PAHs are considerably different from the ones formed by small alkylating agents. For example the dihydrodiol epoxide metabolites of PAHs react predominantly at the exocyclic amino groups of guanine and adenine. The major DNA adduct of the carcinogen, benzo[a]pyrene-7,8-dihydrodiol-9,10-oxide, occurs at N\textsuperscript{2} of guanine [166]. DNA binding basically depends on its molecular structure and functional state (accessibility of nucleophilic target sites) while physiological and biochemical features determine differences in adduct formation among tissues and across species.

DNA adducts caused by PAH metabolites are known to be crucial factors in the aetiology of cancer development. For this reason their presence and formation has been profusely studied. Adducts with benzo[a]pyrene (recognized as a model compound for the PAH group) are the most frequently reported [167, 168], but adducts can also be formed with low molecular weight PAHs, like chrysene, which is a constituent in most mineral oils [169]. A schematic outline of the adduct formation process is outlined in Figure 9.

DNA adducts have been used as a biomarker of exposure to PAHs since the ‘90s. They represent a very important endpoint, being a marker of genotoxicity. Numerous monitoring studies have reported the formation of DNA adduct formation in aquatic organisms (e.g. fish and bivalves) due to exposure to PAHs. For example, Lyons et al [171] and Harvey et al. [172] reported the genotoxic impact of the Sea Empress oil spill accident on different fish species (Lipophorus pholis and Limanda limanda) as well as on invertebrates (Halichondira panicea and Mytilus edulis). The DNA adduct patterns of fish liver exhibited the typical diagonal radioactive zone (DRZ) even 17 months after the spill took place. Detection of DNA adducts has been used to assess the impact of the Erika oil spill along the coasts of French Brittany. To confirm that the DNA adducts were really related to the Erika
polycyclic aromatic hydrocarbons (PAHs) from petroleum, human hepatocyte (HePG2 cells) were exposed to an *Erika* fuel extract. Incubation of HePG2 cells lead to the formation of DNA adducts with similar patterns to the ones observed in the monitoring study using fish (*Solea solea*). These data indicates that human hepatocytes biotransform *Erika* fuel into genotoxic metabolites similarly to hepatic cells of fish and confirmed that the adducts observed in the monitoring study were related to the contamination of the sediment by the oil spill [173].

**Figure 9.** Metabolism of PAH leading to protein and DNA adducts. Figure adapted from reference [170].

It has been shown that DNA adducts persisted in vertebrates species due to the low efficiency of repair systems, representing a parameter for long term exposure [174]. These adducts are very persistent in fish liver [175-178]. French et al. [174] observed a steady increase in DNA adduct levels during a chronic exposure of sole (*Pleuronectes vetulus*) to PAH contaminated sediment for 5 weeks, which were persistent even after a depuration period. The persistency of DNA adducts has been demonstrated also in Atlantic cod (*Gadus morhua*) [38]. In this study, hepatic DNA adducts appeared after 3 days of exposure to low concentration of crude oil (0.06 ppm) and increased steadily during the entire exposure period of 30 days.

Several techniques (e.g. immunoassay, fluorescence assay, gas chromatography-mass spectroscopy (GC-MS), ³²P-postlabelling and mass spectrometry (MS) analysis) have been developed for the analysis of PAH derived DNA adducts. At present, the most sensitive and frequently applied technique in aquatic organisms is the ³²P-postlabelling assay [179]. Its high sensitivity is unique and achieves the determination of 1-100 adducts in 10⁹ nucleotides [180].
The $^{32}$P-postlabelling assay appeared in the early ‘80s and has been applied with a range of protocols in order to detect DNA adducts produced by known carcinogens and complex mixtures [181]. Briefly, the assay involves DNA purification, digestion to normal and adduct-modified 3’-mononucleotides, removal of normal nucleotides (via enzymatic digestion, solvent extraction or chromatographic methods), $^{32}$P-postlabelling at the 5’ position of adducted nucleotides followed by chromatographic separation, detection and quantification (via autoradiography, scintillation counting or phosphor screen imaging analysis) [182]. Following this assay, PAHs cause the appearance of the diagonal radioactive zone (DRZ) (Figure 10) [173,178,183,184].

![Figure 10. Example of the bulky DNA adduct zone (DRZ) typical of a contamination by PAHs detected by the $^{32}$P-postlabelling method (liver sample of fish collected in a PAH contaminated coastal area) (Pampanin unpublished data).](image)

MS/MS analysis has recently emerged as a powerful tool in the detection and structure elucidation of DNA adducts as well as for their quantification at very low concentrations (as often present in biological samples). In fact, electrospray ionization tandem mass spectrometric (ESI-MS/MS) analysis was capable of revealing DNA adducts in different aquatic organisms (e.g. fish (Oreochromis mossambicus) and mussel (Perna perna) soft tissue) [185,186]. This MS/MS approach provided a rapid determination and discrimination of structurally different phenanthrene derived DNA adducts in fish bile samples [186]. This technique is able to detect one modified base in $10^6$-$10^{12}$ unmodified bases [187].

Development of methodologies to detect DNA damages induced by PAHs is of constant concern in aquatic ecotoxicology. Direct chemical methods, such as high performance liquid chromatography with electro-chemical detection (HPLC-EDC), GC-MS and the $^{32}$P-postlabelling, are highly sensitive and specific [180,188], however, they are very time and money consuming. A number of antibodies have been generated against carcinogenic products of DNA modifications, including those generated by PAHs. Immunoassays (immunohistochemistry or ELISA) are routinely employed to detect DNA adducts in humans, while the use of such approaches is more limited in aquatic species [189]. An immunoperoxidase method for revealing 7,8-dihydro-8-oxodeoxyguanosine (8-oxo-dG) in marine organisms has been described [189]. This work was also followed by the use of immunofluorescence and antibodies for DNA adduct detection in both vertebrates (fish,
Anguilla anguilla) and invertebrated (mussel, Mytilus galloprovincialis). The immunohistochemical approach demonstrated a good selectivity, low cost, is easy to perform and readily allowed the analysis of a large number of samples. Nevertheless, it does not reach the high sensitivity of other methods [190].

7. Protein adducts from polycyclic aromatic hydrocarbons

As discussed earlier (vide infra) CYP1A derived PAH metabolites have a high affinity to nucleic acids and proteins, which may result in adduct formation (Figure 11). It is likely that PAH protein adducts are formed after an initial docking, viz. protein-ligand interaction, of the PAH metabolite to the protein followed by reaction with nucleophilic sites in the vicinity of the docking site [191,192]. This has been shown to be the case for human serum albumin, which is predominantly alkylated at histidine146 by diol epoxides of fluoranthene and benzo[a]pyrene [193]. It is highly likely that the same type of mechanism is operating in animals and fish.

![Figure 11. General mechanism for PAH protein adduct formation.](image)

Naturally, adduct formation between PAH metabolites and human proteins and in particular human hemoglobin and serum albumin have been very well documented [170,192-199]. However, there are a few studies concerning protein adducts in fish. Plasma albumin adducts have been isolated from two species of fish (Brook trout (Salvelinus fantinalis) and Arctic charr (Salvelinus alpinus)) [200]. In the studies conducted by Padrós and Pelletier, which were conducted by injection of benzo[a]pyrene, it was found that only the (+)-anti-diol-epoxide of benzo[a]pyrene generated adducts with serum albumin. In that study it was also found that there was no accumulation of the adduct upon repeated injections, thus indicating a relatively short half-life of less than two days for serum albumin.
in fish. In humans the half-life of serum albumin has been reported to 20 days [201]. In fish the presence of this adduct would be an indication of a very recent exposure to benzo[a]pyrene, while in humans this would also function as a marker of longer term exposure to the PAH. It has been found that the point of adduct formation between human serum albumin and benzo[a]pyrene anti-diol epoxide is dependent on the stereochemistry of the PAH metabolite [194]. The (+)-enantiomer generates a carboxylic ester adduct with Asp^{187} or Glu^{188} and that the (-)-enantiomer forms an adduct with His^{146}.

The different isoforms of hemoglobin present in different species results in the formation of different adducts. For example, rat hemoglobin possesses a reactive β-cysteine in position 125 not present in human hemoglobin [202]. *syn* And *anti* fluoranthene diol-epoxides form adducts with this particular cysteine in rats. The presence of different isoforms of the same protein in different species results in the possibility of generating different adducts for the same PAH in the different species. The point of adductation most likely reflects on the proteins ability to function. Thus, the adduct formation of a specific protein might affect one species more severely than another.

8. Future perspective

Environmental research related to PAHs has to date, with a few exceptions, predominantly been concerned with finding metabolites of the compounds and detecting the presence of PAH DNA adducts. However, based on the discussion herein it is clear that the next step has to be towards analysis that can provide clear answers regarding the stereochemical outcome of the oxidation processes taking place *in vivo*. By such means it is easier to evaluate the toxicity of the various PAHs. This is a rather large task since different species metabolize PAHs differently resulting in dissimilar distributions between the stereoisomers.

PAH protein adducts have been studied extensively for humans (*vide supra*) and rodents, however, for fish and other aquatic animals this topic is barely touched upon. Studies of adduct formation in fish will further aid the evaluation of the toxicity of the different PAHs. In the PAH protein adduct studies that have been conducted on other species we have seen that the point where the adduct is formed in a specific protein varies from species to species. Generating new knowledge as to where adducts are formed with the same PAH in the same protein in other species might in addition to providing increased knowledge as to the impact of adduct formation also possibly generate new interesting research questions. Hemoglobin in fish has a very short half-life so adduct formation on hemoglobin might not have such a great health impact on the fish. However, other less abundant proteins in the blood are also most likely susceptible to adduct formation with PAH metabolites. Detecting the proteins affected and determining the site of adduct formation will aid in the overall judgment of the toxicity of the PAH responsible for the adduct formed. Adducts with proteins present in the bile may also be of value in assessing the toxicity of PAHs.

In human health care proteomics has for some time been extensively used for diagnostics [203,204] and these techniques are also slowly making their way into ecotoxicology [205,206]. It has been found in human amniotic epithelial cells exposed to anti-7,8-
dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, a compound that causes adduct formation on DNA and oxidative damage on DNA, resulting in alternations of the expression of three proteins [207]. This result highlights that proteomics and the study of expression rates of particular proteins can be a powerful method in the future in order to determine if marine animals have been exposed to PAHs present in oil.

**Author details**

Daniela M. Pampanin  
*Biomiljø, International Research Institute of Stavanger, Mekjarvik, Randaberg, Norway*

Magne O. Sydnes  
*Faculty of Science and Technology, University of Stavanger, Stavanger, Norway*

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Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds found in petroleum and are known to be present in the aquatic environment. They can influence aquatic ecosystems by interacting with various biological and chemical processes. The metabolism of PAHs by cytochromes P450 (CYPs) is an important area of research, as these enzymes play a crucial role in the biotransformation of PAHs into more reactive species.

1. **Polycyclic Aromatic Hydrocarbons as Constituents of Petroleum: Presence and Influence in the Aquatic Environment**


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benzo[a]pyrene, genzo(k)fluoranthrene and 7H-dibenzo(c,g)carbazole. Mutat. Res. 427:135-145.


