12 Identification of Hydrocarbons in Biological Samples for Source Determination

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12.1 Introduction

Aquatic fauna are often examined for evidence of hydrocarbon exposure, as it may pose a direct threat to their health. Whether petroleum-derived hydrocarbons are released from chronic sources or from isolated, intermittent spills, biological tissues are routinely sampled and analyzed to evaluate the spatial extent and persistence of pollution, as well as the potential for adverse effects. Evidence obtained from these analyses can be paramount in litigation, where estimates of lost resource value associated with the extent of biological injury can form the basis of damage claims. Such estimates are rarely straightforward because multiple hydrocarbon sources must nearly always be distinguished, making it difficult to establish a clear link between the evidence of biological impact and the sources responsible for it.

In addition to the challenges associated with fingerprinting neat product samples following environmental release, analysis of hydrocarbons in biological tissues must also account for the route of hydrocarbon uptake and biotransformation. The four most important uptake pathways are (1) physical coating on the external surfaces of biota, possibly followed by passive partitioning through the skin, (2) ingestion of whole particulate oil or oil-contaminated prey, (3) passive partitioning of dissolved hydrocarbon components into biological tissues of aquatic biota, and (4) inhalation of hydrocarbon vapors. Absorbed hydrocarbons may then undergo biochemical transformation, depending on the metabolic capacity of the organism involved. Invertebrates are often less well-equipped to metabolize hydrocarbons compared with vertebrates, but there is considerable phylogenetic variability in the distribution of this competence, even within phyla (Livingstone, 1998). When it can be shown that hydrocarbon composition changes mediated by partitioning among phases or by biochemical transformations are not important, then biota often provide a convenient sampling matrix for evaluating the extent, persistence, and source of hydrocarbon contamination.

Clearly, the utility of tissue hydrocarbon analyses depends on the questions asked. When biota are sampled and analyzed to evaluate biological exposure or injury, the same data may provide evidence that constrains prospective hydrocarbon sources. As physical and biochemical processes can modify an already complex hydrocarbon profile, it is important to establish the limitations of data derived from the chemical analysis of biota

with respect to the issue at hand. In some cases, analyses of biota may furnish evidence for source identification that is comparable with sediments or neat product for hydrocarbon source identification. In others, only rough estimates of hydrocarbon concentrations in the aqueous environment may be inferred from concentrations found within organisms. Even when the hydrocarbon fingerprint of the source is extensively modified by partitioning and biochemical transformation, biochemical evidence of hydrocarbon exposure may provide spatial and temporal patterns that implicate particular sources or eliminate others. In every case, all processes having the potential to alter the original hydrocarbon source signature must be considered carefully if the resulting evidence is to be applied defensibly.

Our objective here is to provide a succinct overview of the main processes that affect the interpretation of hydrocarbons in biota, with respect to inferences regarding hydrocarbon source. The primary issue in this endeavor is determining when definitive indicators of the hydrocarbon source are preserved in the biological matrix sampled. When they are, then inferences regarding sources may proceed along the lines that have been developed for fingerprinting environmental samples affected only by physical weathering processes, the subject of extensive literature discussed elsewhere in this volume (e.g., Chapters 3 and 13). When they are not, the associated sample collection information may still furnish evidence regarding prospective sources, albeit less conclusively. We therefore begin with a summary of the physical processes altering hydrocarbon composition that are associated with each of the main routes of hydrocarbon exposure for biota, which determines the degree of source-specific information retained by accumulated hydrocarbons. This is followed by a discussion of the biochemical pathways that may transform the hydrocarbons accumulated, which may stimulate biochemical responses such as cytochrome P450 induction that can be very sensitive indicators of exposure. These pathways typically reflect biochemical adaptations to mitigate the toxicity of accumulated hydrocarbons, so their relation to hydrocarbon mechanisms of toxicity is also presented. Finally, many of these principles are illustrated by the comprehensive studies of the *Exxon Valdez* oil spill (see ahead and Chapter 15). While our coverage of the relevant literature is far from exhaustive, we have tried to include references to studies and reviews that have been helpful in gaining a broader understanding of the processes involved while providing ready identification of more focused studies.

12.2 Determination of the Primary Route of Hydrocarbon Accumulation by Biota

As soon as a petroleum-derived product is released into the environment, its composition changes in response to physical and biological processes, including evaporation, dissolution, microbial oxidation, and photooxidation, collectively referred to as "weathering" (Garrett et al., 1998; Ezra et al., 2000). Initially, evaporation is the key process, followed by dissolution if the spilled material is in contact with water. While microbial processes require more time, they can lead to profound compositional changes that are more difficult to predict because isomer-specific degradation rates vary among microbial species (e.g., see Chapter 11 herein), so composition changes caused by microbial degradation depend on the composition of the ambient microbial flora. Photooxidation is inherently slower, affecting mainly the polycyclic aromatic hydrocarbons (PAHs, herein used to include heterocyclic aromatics) on oil surfaces exposed to strong sunlight and high oxygen concentrations (Hansen, 1977).

Substantial changes in the hydrocarbon profile, such as the loss of the most volatile components, can result from evaporation from thin oil films on time scales of hours to days (Payne et al., 1984). For crude oils, evaporation may account for losses of ~25% or more, with considerably greater losses for lighter distillates, but less for heavy residual fuels such as bunker oils (Fingas, 2004). Due to their

lower vapor pressures, evaporative losses of less volatile components are sometimes dismissed as negligible but can be substantial. For example, when the ratio of surface area to volume of an oil $(A/V)_{oil}$ is large and the oil is exposed to the atmosphere for long periods (years), considerable proportions of semivolatile components may ultimately be lost through evaporation (Short and Heintz, 1997).

Dissolution is a more gradual process than evaporation because the aqueous boundary layer adjacent to the oil parcel surface provides more diffusive resistance to the flux of dissolved components away from the oil. Dissolution also results in different phase-partitioning compared with evaporation. Evaporative component losses are determined primarily by vapor pressure. For example, naphthalene, with a molecular mass of 128, has a vapor pressure of 3.95×10^{-4} atm[1] at 25°C (Hinckley et al., 1990), equivalent to a gas-phase concentration of 1.62×10^{-5} mole/L, whereas nonane, an alkane of the same molecular weight, has a much higher vapor pressure[2] (6.07×10^{-3} atm) and gas-phase concentration (2.49×10^{-4} mole/L). The lower vapor pressure of naphthalene is due to the stronger attractive Van der Waals force associated with its less tightly bound aromatic π-electrons. Hence, the aliphatic hydrocarbons in petroleum products have a much greater tendency to be lost through evaporation compared with aromatic hydrocarbons of similar molecular masses. Just the opposite situation occurs with dissolution. The solubility of naphthalene[1] in water at 25°C is 9.44×10^{-4} mole/L (Shiu et al., 1988), or ~58 times greater than its "solubility" in air, while the solubility of nonane is 1.72×10^{-6} mole/L (McAuliffe, 1969), which is *lower* than its solubility in air by a factor of ~150. This comparison demonstrates that water acts like a sponge for aromatic hydrocarbons, but as a lid for aliphatics, when compared with the atmosphere. As a result, the water-soluble fraction (WSF) of oil is enriched with the more soluble aromatic hydrocarbons but deficient in aliphatics.

Microbial oxidation may cause dramatic changes in oil composition on time scales of days to years following release to the environment (e.g., see Chapter 11 herein), depending on the favorability of growth conditions. Warm temperatures, a steady supply of essential nutrients, and high initial populations of competent microbes are the most important factors promoting rapid and sustained oxidation rates. The *n*-alkanes are the most readily oxidized substrate in petroleum products, followed by branched alkanes, unsubstituted PAH, and alkyl-substituted PAH (Pirnik, 1977; Schaeffer et al., 1979; Payne et al., 1984; Wang et al., 1996). Other components of oil, even including some of the most persistent compounds such as the complex alicyclic hopanes, other triterpanes, and steranes, are susceptible to eventual degradation by microbial oxidation although at much slower rates (see, e.g., Wang et al., 2000).

The weathering processes summarized above lead to hydrocarbon degradation patterns that are nearly always observed following oil spills. First, evaporation rapidly purges the most volatile hydrocarbons, including aliphatics with molecular weights below *n*-dodecane, and the monocyclic aromatics (especially benzene, toluene, ethyl-benzene, and the xylenes, or BTEX compounds). These compounds are consequently lost to the atmosphere within the first week or two of a spill (e.g., Payne et al., 1984). Second, if the spilled oil is dispersed in an oxic environment, the *n*-alkanes are consumed by microbes relatively rapidly. The more resistant isoprenoids such as pristane and phytane remain somewhat longer as resolvable peaks on aliphatic chromatograms, usually associated with a substantial unresolved complex mixture (UCM), which appears as a hump (see, e.g., Payne et al., 1984; Boehm et al., 1987). Indeed, the ratio of *n*-octadecane/phytane has often been used as a measure of the extent of aliphatic

[1] Values are for the subcooled liquid, i.e., the value that would be observed if the compound were in the liquid state at 25°C (see Shiu et al., 1988).

[2] Estimated from application of the Clausius–Clapeyron equation to vapor pressure data tabulated in Weast (1977).

biodegradation since phytane is rarely found apart from petroleum-derived products (Dean and Whithehead, 1961; Blumer and Snyder, 1965).

Finally, if oil is in an aquatic environment, the water will extract PAH at rates that decrease with increasing number of rings and degree of alkyl substitution. This pattern is the result of the effects of molecular volume on solubility (McAuliffe, 1966). At equilibrium, the extent of PAH loss from oil is determined by the distribution coefficient K of the PAH and the relative volumes of oil and water involved. Shiu et al. (1988) show that the equilibrium concentration C_w of a PAH in water is approximately $C_{so}/(K + Q)$, where C_{so} is the initial concentration of the PAH in the oil, K is ratio of the equilibrium concentration of the PAH in the oil (C_o) and C_w, and Q is the water-to-oil volume ratio V_w/V_o. The distribution coefficient K is rarely measured, but may be approximated by the octanol-water distribution coefficient K_{ow}. For a 0.1-mm-thick oil slick in equilibrium with a 10-m mixed layer of water beneath it, $Q = 100{,}000$, implying that more than 50% depletion will occur for PAH with K_{ow} smaller than this value. This would include all the naphthalenes bearing as many as three alkyl carbon atoms, and phenanthrenes with as many as one, whereas dissolution losses of four-ring PAH would be considerably smaller. At smaller values of Q, the extent of PAH loss from oil is determined principally by the distribution coefficient K. The value of K increases rapidly with increasing molecular volume, leading to aqueous PAH distribution patterns that are strongly dominated by the most soluble components. Conversely, the aqueous distribution pattern will approach that initially observed in the oil when Q becomes large relative to K of the least soluble PAH (which may be $\sim 10^6$). Under these conditions, there is sufficient water available to extract nearly all the PAH initially present in the oil. However, despite the large water-to-oil volumes that may be applicable during oil spills, such extreme depletion is rarely observed. This is because oil releases usually occur in open systems that are far from equilibrium, so dissolution kinetics are also important. Hence, in addition to being less soluble, larger PAH also dissolve more slowly, further enhancing relative concentrations of the lower-molecular-weight PAH in the aqueous phase.

Selective PAH loss from dissolution, often called "water-washing," is the dominant cause of changes in the PAH profile of spilled oil and follows a course that may be predicted from the initial PAH composition of the oil and the molecular surface area of the PAH. A simple model of this process (Short, 2002) accurately reproduces the dominant PAH weathering patterns observed following oil spills. For example, application of this model to the PAH composition of *Exxon Valdez* cargo oil matched the analytical results of nearly all of the hundreds of samples of oiled sediments and mussels within analytical precision (Short and Heintz, 1997), implying that other PAH weathering processes were minor compared with evaporation and dissolution after that spill. The PAH weathering sequence predicted by the model is depicted in Figure 12-1. This characteristic pattern of PAH losses produces a complementary pattern of dissolved PAH in the receiving water, illustrated in Figure 12-2, calculated from the difference in PAH concentrations represented in the two uppermost panels of Figure 12-1. The enrichment of the more readily soluble PAH is apparent and reflects the preferential partitioning of PAH into water discussed above. Collectively, these weathering processes provide crucial guidance for deducing the exposure route based on hydrocarbon concentration patterns observed in biota.

An uptake pathway involving accumulation of whole oil by an organism is indicated if the hydrocarbon distribution pattern matches the source oil, allowing for weathering and provided the organism's metabolism does not alter the pattern further. Oil adhering to the external surfaces of animals such as marine mammals and birds is an obvious example. Less obvious is oil accumulated by invertebrate suspension feeders such as mussels (*Mytilus sp.*) or in the intestinal tracts of fish. Chemical hallmarks of whole-oil accumulation include a complete set

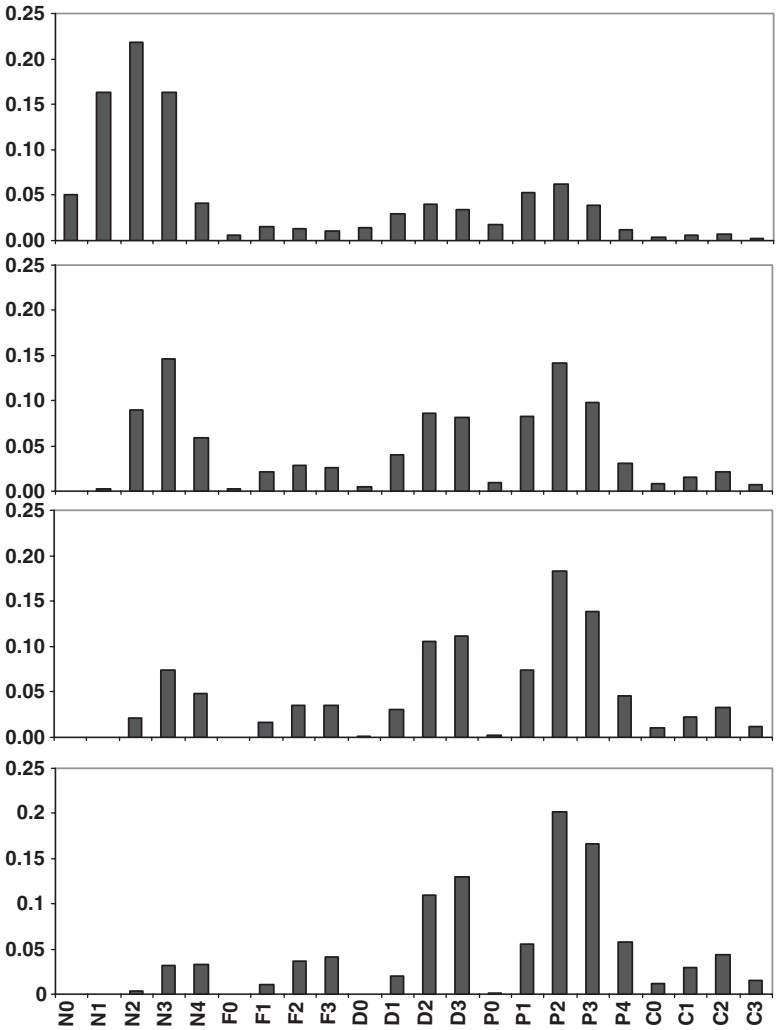

Figure 12-1 Typical weathering sequence of PAH in Alaska North Slope crude oil. N = naphthalene, F = fluorene, D = dibenzothiophene, P = phenanthrene/anthracene, C = chrysene; numbers indicate substituent alkyl carbon atoms. Weathering increases from top panel (unweathered oil) to bottom panel (moderately weathered oil). Abscissa: proportion of total PAH.

of petroleum biomarkers[3] (e.g., hopanes, other triterpanes, and steranes) that have ratios matching the corresponding ratios of the source oil, a water-washed PAH distribution pattern comparable with those shown in Figure 12-1 (reflecting the weathering state of the oil when ingested), a prominent aliphatic and aromatic UCM, and often a variable profile of *n*-alkanes, perhaps with pristane and phytane prominent. When these criteria are met, the source identification fingerprinting methods applicable to whole-oil samples (see Wang et al., 1999, for a review) may be confidently

[3] The term "biomarker" is used in two different ways in oil pollution studies. Petroleum biomarkers, sometimes called "molecular fossils," are alicyclic hydrocarbons that persist on geologic time scales and are usually associated with specific evolutionary events such as the appearance of flowering plants. Biological biomarkers refer to biochemical responses to pollutant exposure that are readily identifiable, such as the detoxification pathways involving cytochrome P450 enzymes.

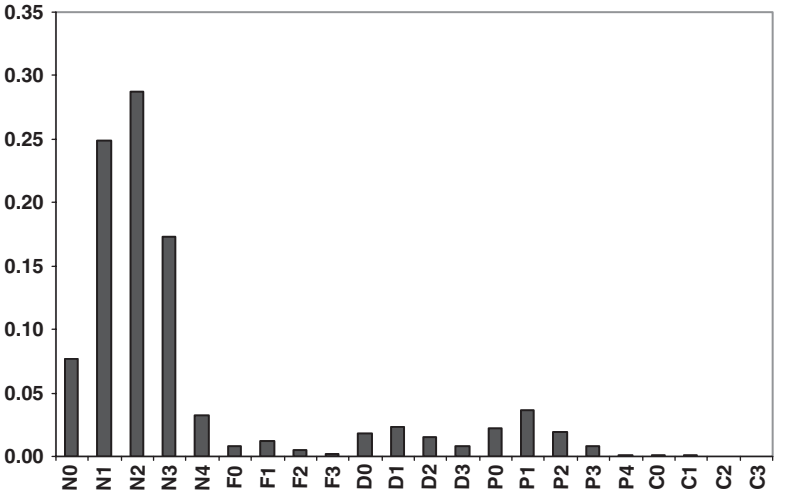

Figure 12-2 Relative composition of aqueous PAH dissolved from initially unweathered Alaska North Slope oil. Abbreviations and axes as in Figure 12-1.

applied to biological samples. Indeed, such samples are often invaluable in source identification.

Hydrocarbon results for mussels collected two months following the *Exxon Valdez* oil spill provide an example of hydrocarbon distribution patterns indicative of whole-oil accumulation (Figure 12-3). Comparison of PAH patterns of surface oil collected 11 days following the spill with those of mussels collected about six weeks later clearly shows the water-washed PAH pattern depicted in Figure 12-1 in the mussels, but phytane as a proportion of total PAH is little changed. Because the aqueous solubility of phytane is negligible, it serves as a petroleum biomarker for the weathered whole oil.

Much less source information is available from organisms that accumulate hydrocarbons from the dissolved phase. Uptake of dissolved hydrocarbons involves passive partitioning between the aqueous phase of the exposure medium and the lipid compartment(s) of the organism. An organism exposed to the dissolved-phase PAH distribution illustrated in Figure 12-2 will acquire a PAH burden resembling it. The higher-molecular-weight PAHs that are less readily extracted from the oil by the water are less available for subsequent partitioning into biotic lipids. Hence, mussels exposed to water-soluble fractions of crude oil typically contain scant four-ring PAH, for example, because so little of these partition into the water from the oil. After partitioning from crude oil via an intermediate aqueous phase into biota, PAH distributions resemble patterns that result from diesel oil, since the initial dissolution process truncates the higher boiling and less soluble PAH in the crude oil (which are removed during the refining process in diesel oil). Consequently, the resulting hydrocarbon signature in biota that accumulates only dissolved hydrocarbons will usually retain little information regarding the source.

Of course, aquatic biota may be simultaneously exposed to both dispersed oil droplets and their dissolved components, but this is most likely soon after (and close to) a spill event. Once an oil–water dispersion of droplets is created, it will promote dissolution of the soluble components because of the increase of $(A/V)_{oil}$, and the droplets will respond to buoyancy forces. The dissolved components, though, will not, which usually leads to separation of oil droplets from the most soluble oil components in the affected water masses.

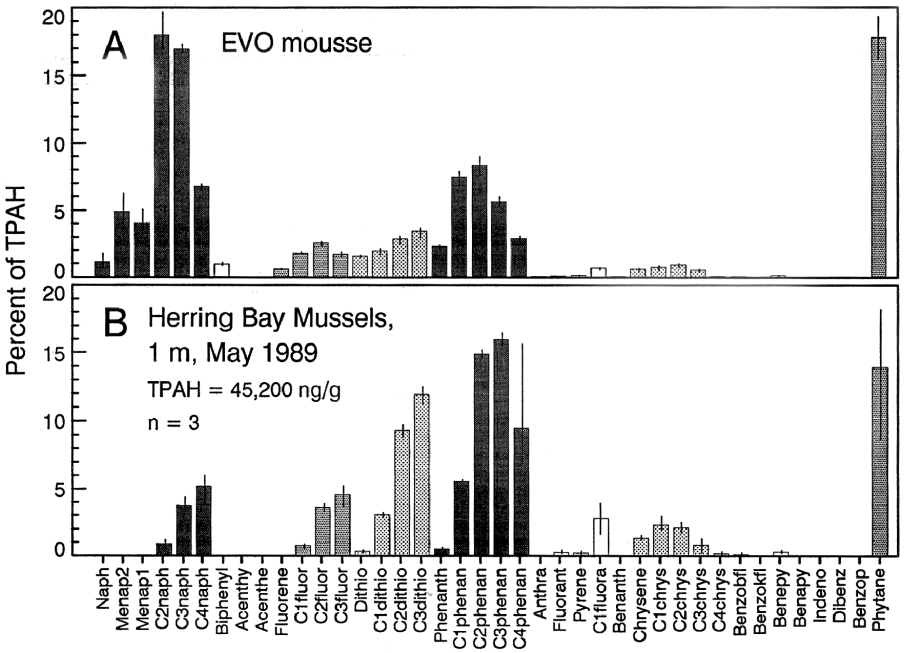

Figure 12-3 Relative abundance of PAH and phytane in (A) *Exxon Valdez* oil mousse 11 days following the spill, and (B) in mussels collected six weeks later. Note the PAH weathering evident in the mussels compared with the oil (compare with Figure 12-1), and the prominent phytane in both, indicating ingestion of whole weathered oil droplets by the mussels. Abbreviations: Naph = naphthalene, Menaph = methylnaphthalene, Acenthy = acenaphthylene, Acenthe = acenaphthene, Fluor = fluorene, Dithio = dibenzothiophene, Phenan = phenanthrene, Anthra = anthracene, Fluorant = fluoranthene, Benanth = benzo[*a*]anthracene, Chrys = chrysene, Benzobfl = benzo[*b*]fluoranthene, Benzokfl = benzo[*k*]fluoranthene, Benepyr = benzo[*e*]pyrene, Beneapyr = benzo[*a*]pyrene, Indeno = indeno[1,2,3-*c,d*]pyrene, Dibenz = dibenzo [*a,h*]anthracene, Benzop = benzo[*g,h,i*] perylene (from Short and Harris, 1996b).

Considerably less is known about tissue hydrocarbon patterns that characterize exposure pathways involving vapor inhalation and absorption across the skin. These pathways are thought to be important for marine mammals (Engelhardt, 1987; Geraci and St. Aubin, 1987; Spraker et al., 1994), but are difficult to study. Inhalation of hydrocarbon vapors may lead to reversible narcosis when not fatal, so evidence of exposure is usually either ephemeral or associated with a carcass on the sea floor. PAHs that are absorbed through the skin (or through the intestinal wall) are subject to the efficient biochemical detoxification pathways found in mammals, which may obscure evidence of dermal absorption. Although the biochemical detoxification responses may themselves become the primary indication of exposure, in these cases almost all information associated with hydrocarbon distribution patterns that reflect those of the original hydrocarbon sources is lost. A summary of these pathways is presented in the next section.

12.3 Catabolic Degradation of Hydrocarbons Accumulated by Biota

12.3.1 Catabolic Degradation of PAH

Catabolism is the breakdown of complex substances into simpler molecules. At least some capacity to catabolically degrade PAH is widely distributed among the animal kingdom (Livingstone, 1998), resulting from the need to detoxify poisons produced by prey. The evolutionary competition between predators and prey has produced chemical defenses

of generally increasing sophistication among prey, especially plants, which has in turn selected for detoxification pathways in predators that are commensurately sophisticated. These detoxification pathways are more complex and efficient in more evolutionarily advanced organisms, which leads to large differences in detoxification rates among organisms. Before considering these differences, it will be helpful to summarize the basic molecular biology of the most important PAH detoxification pathways, which involve the cytochrome P450-dependent monooxygenases.

Cytochrome P450 enzymes are a superfamily of heme-containing proteins that catalyze the oxidation of their substrates and are so named because their complex with carbon monoxide has an absorption maximum at 450 nm (Ortiz de Montellano, 1995). Some of these enzymes are inducible by their substrates, facilitating a biochemical adaptation to changing environmental conditions (Denison and Whitlock, 1995). One of these, cytochrome P450 1A (CYP 1A hereafter), catalyzes the oxidation of planar aromatic molecules including PAH, dioxins, and some polychlorinated biphenyls (PCB) (Nelson et al., 1996). In most species, this protein is found at low constitutive levels in the cytosol due to its suppression by a repressor protein. In the presence of an inducer, however, both the derepression and activation of transcription by the aryl hydrocarbon receptor (AhR) are involved. The AhR is normally complexed in a 1 : 2 ratio with heat shock protein (hsp 90), which dissociates on binding of a planar aromatic ligand to the AhR. The activated AhR may cross the nuclear membrane, where it can bind with the aryl hydrocarbon nuclear translocator protein (ARNT). The resulting heterodimer then binds to DNA regulatory sequences located upstream of the CYP 1A gene, thus upregulating its expression (Whitlock, 1993). This cascade of events stimulates the production of messenger RNA, followed by protein translation on the endoplasmic reticulum to produce CYP 1A in the cytosol (Figure 12-4). The CYP 1A catalyzes several types of oxidative reactions making its substrates, such as PAH or other planar aromatics in the cytosol, more water-soluble and readily excreted. These reactions can introduce an epoxy group to the aromatic ring, which may then undergo further metabolism to form a diol or diol epoxide (Parkinson, 2001). Depending on the parent compound, the metabolism of PAHs can increase their toxicity by activating the toxin, leading to protein and DNA adducts, and possibly cancer (Varanasi et al., 1986) or other adverse effects (Guengerich and Liebler, 1985). The substrate specificity of CYP 1A is less restrictive than is the ligand-AhR binding specificity, so that not all PAHs are effective inducers of CYP 1A (Barron et al., 2004), but once induced, CYP 1A may oxidize a broader spectrum of PAH (Hawkins et al., 2002).

This initial oxidation by CYP 1A is often referred to as phase I metabolism to distinguish it from phase II metabolism, which involves condensation of the hydroxylated aromatic with a sugar or other highly soluble compound to increase the water solubility while decreasing the toxicity of the phase I metabolite. In vertebrates and in some invertebrates, the same ligand-AhR binding that triggers CYP 1A production also activates other genes that code for phase II catabolic pathways (Foureman, 1989). These include glutathione *S*-transferase, uridine diphosphate-glucuronyl and -glucosyl transferase, sulphotransferase, and amino acid conjugases (Whitlock, 1993).

These phase I and II detoxification pathways are fully developed in the vertebrates, but are much less so in the more primitive phyla. For example, an inducible CYP 1A system is absent in cnidarians (Kaji et al., 1983; Newman et al., 1990) and probably in sipunculid worms (Lee, 1981) and is generally less active in annelids, mollusks, and echinoderms when compared to crustaceans, fish, and vertebrates (Lee, 1981; Livingstone, 1998; Chaty et al., 2004). When present in mollusks, echinoderms, and species of other less advanced phyla, CYP 1A activity is localized in the digestive tract (Lee, 1981; Livingstone, 1998) but is more concentrated in the hepatopancreas of crustaceans. In vertebrates, CYP 1A

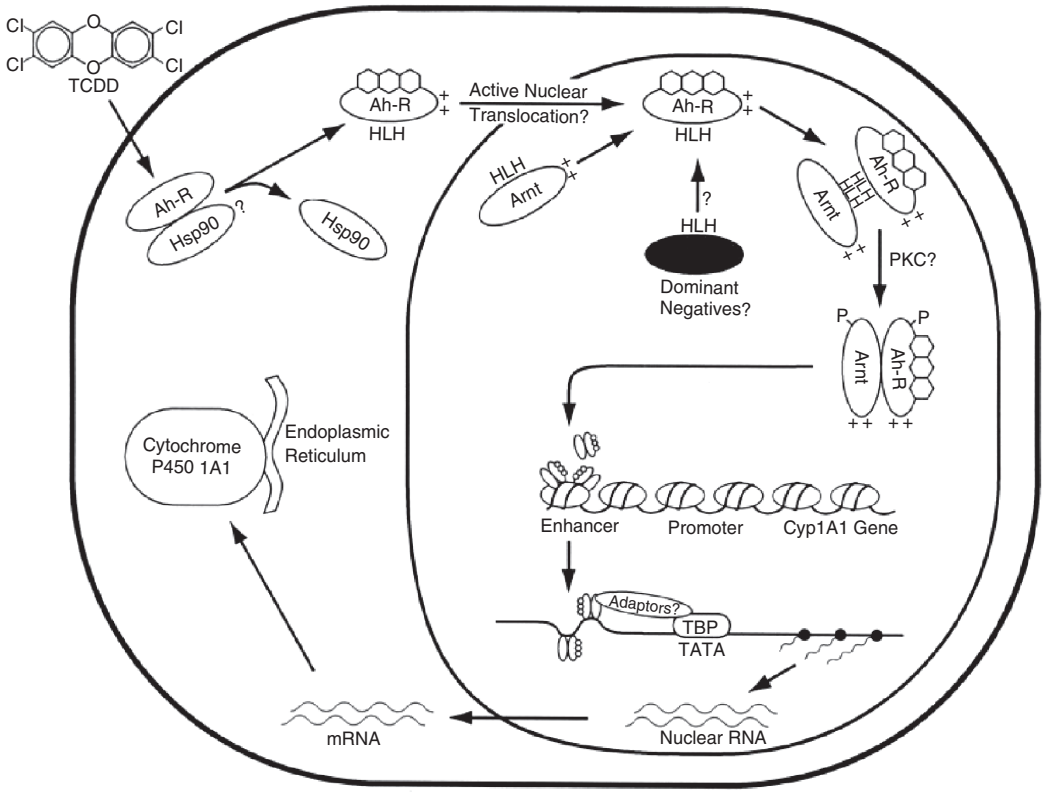

Figure 12-4 Cytochrome P450 1A induction pathway (from Whitlock, 1993; see text in section 12.3.1 for abbreviation).

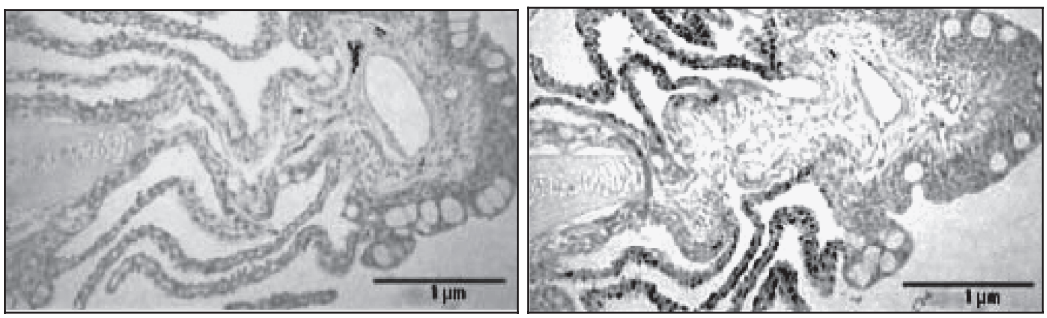

Figure 12-5 Gill epithelial cells under normal conditions (left), and under CYP 1A induction (right) (from Malins et al., 2004).

activity can be found in a broad array of vertebrate cell types, including gill epithelial cells (Figure 12-5), with the liver as the focus of CYP 1A activity (Livingstone, 1998).

Almost every aspect of the CYP 1A induction sequence has been used as a basis for evaluating induction, including antibody reactivity with the CYP 1A protein, detection of the mRNA sequence, and direct assay of catalytic activity (Stegeman and Hahn, 1994; Bucheli and Fent, 1995; Chaty et al., 2004). Of these, direct assay of catalytic activity is most widespread, due to its specificity, relative ease, and low cost. The most popular assay is based on

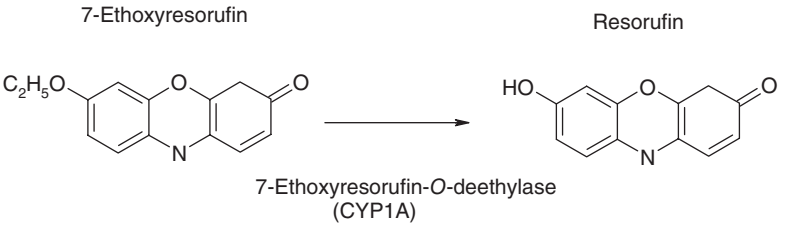

Figure 12-6 Catalysis of 7-ethoxyresorufin to resorufin by cytochrome P450 1A (CYP 1A). Resorufin is fluorescent, and its production rate is the basis for the EROD assay for CYP 1A activity.

the de-ethylation of 7-ethoxyresorufin to form resorufin (Figure 12-6), usually denoted as the EROD assay (Whyte et al., 2000). The substrate is mixed with a microsomal suspension isolated from homogenized liver tissue, along with NADPH, Mg^{2+}, and a buffer. The resorufin produced may be analyzed by absorption spectrophotometry or by fluorescence, with excitation at 530 nm and emission at 580 nm.

Various aspects of the CYP 1A induction sequence provide the basis for a suite of biological biomarkers indicative of exposure to planar aromatics, including PAH. Measurement of high specific CYP 1A activity (i.e., picomoles of resorufin/mg protein/min) is one of these, but several others are in current use in pollution studies [see reviews by Stegeman and Hahn (1994), Bucheli and Fent (1995), and van der Oost et al. (2003)]. The amount of CYP 1A enzyme induced may also be measured immunochemically by binding with functionalized antibodies (e.g., Smolowitz et al., 1991), by enzyme-linked immunosorbent assay (ELISA), or by other related immunological techniques (see reviews by Stegeman and Hahn, 1994, and by Bucheli and Fent, 1995). Detection of the mRNA encoding the CYP 1A protein may be separated by electrophoresis and detected with a complementary oligonucleotide sequence (northern blot analysis; Bucheli and Fent, 1995). Products of both phase I and phase II metabolism accumulate in the bile of vertebrates, which is a rather convenient compartment to sample in fish. Because these metabolites retain the intact PAH ring structure, they are fluorescent, making them readily detectable. Hence, high-performance liquid chromatography analysis of fluorescent aromatic compounds in biliary fluid can distinguish 2–3-ring, 3–4-ring, and 4–5-ring PAH (Krahn et al., 1992), which may provide information regarding the source of the PAH exposure. Electrophilic PAH metabolites form covalent adducts with DNA, which may be detected using ^{32}P radiolabeling methods (Varanasi et al., 1989), and which provide direct evidence of DNA damage that may lead to carcinogenesis and other symptoms. Another biological biomarker of chromosome damage is the electrophoretic separation of DNA, termed the "comet assay." Broken strands of DNA produce skewed migration patterns that resemble comets in appearance (Fairbairn et al., 1995). However, while very sensitive, the comet assay is less specific because agents other than polycyclic aromatics can cause chromosome breakage.

12.3.2 Effects of Catabolism on PAH Accumulation, Persistence, and Depuration

Establishing a link between evidence of exposure to hydrocarbons and their potential source requires careful consideration of numerous factors. These include the age, life stage, gender, reproductive status, and nutritional status of the target species, the hydrocarbon exposure route, and the characteristics, as well as idiosyncrasies, of the biological measurements involved. Hydrocarbons accumulated as whole-oil droplets (usually as microdroplets)

by suspension feeders with little, if any, capacity to biotransform these hydrocarbons retain considerable information regarding sources. In vertebrates such as fish, which may rapidly metabolize hydrocarbons, the main value biological biomarkers of PAH exposure will usually be as evidence that the organism has been exposed to compounds that can elicit the anticipated effects. Spatial patterns of the intensity of biological biomarker responses in relation to the suspected source may bolster a case, as may temporal patterns, especially if data are available prior to the suspected incident at both control and impacted sites, permitting use of statistically powerful "before/after-control/impact" (BACI) sampling designs (Green, 1979). But in all these applications, it is necessary to have an appreciation for the characteristic timescales of hydrocarbon accumulation, biological biomarker induction, depuration, and persistence of biomarker responses, which are also dependent on target species and exposure route.

Mussels of the genus *Mytilus* are by far the most widely used organisms for assessing marine pollution and oil spills, in part because they are sessile suspension feeders with a limited capacity for altering hydrocarbon distribution patterns when exposed to concentrated pollution sources (i.e., oil spills). These bivalves filter on the order of 1 L of seawater per hour (Foster-Smith, 1975) and capture particles as small as 2 μm (Vahl, 1972). Consequently, their uptake of dispersed oil droplets is very efficient. Mussels may accumulate hydrocarbons associated with dispersed whole oil in excess of 1000 times exposure levels (Fossato and Canzonier, 1976), compared with increases on the order of 20- to 80-fold for 2- to 3-ring PAH when dissolved (Hansen et al., 1978). An important consequence of this effectiveness is that ingestion of oil droplets may rapidly overwhelm the limited ability of mussels to depurate or metabolize these hydrocarbons, thereby preserving source information contained in hydrocarbon distribution patterns. Other advantages of mussels include their large size, making them easy to collect and process for analysis; their relatively high tolerance to oil pollution (Smith, 1968), so they do not usually function abnormally when exposed; and a cosmopolitan distribution. Also, mussels provide a time-integrated sample of their exposure, which captures transient pulses of hydrocarbons that might easily be missed by discrete water sampling. As a result of these advantages, there have been a wealth of studies that have employed them, which facilitates comparisons with other pollution incidents (Bayne, 1976; Donkin et al., 2003; Aarab et al., 2004).

Mussels do have their disadvantages. Their availability may be limited or nonexistent in the desired locations; the variability of accumulated hydrocarbons among identically exposed individuals may be substantial, due to differences in size, lipid content, and reproductive state (and the reproductive state is neither obvious without sacrifice of the animal nor necessarily seasonally predictable); and their pumping rate depends strongly on temperature and food supply (Bayne, 1976). But these disadvantages are all manageable and are usually far outweighed by the convenience of sampling, straightforward and well-characterized chemical analysis methods, and their propensity to preserve hydrocarbon source information that derives from their preferential accumulation of particles (which may be oiled) and scant ability to metabolically transform hydrocarbons.

Compelling evidence of mussels' ability to preserve information regarding hydrocarbon source was demonstrated by a study of the *Exxon Valdez* oil spill, where over a thousand samples collected during a 3-year period following the incident showed PAH distribution patterns that were not distinguishable from those in oiled sediments (Short and Heintz, 1997). The many advantages of using mussels have led to their selection as the marine monitoring species of choice when practical. Mussels have also been of fundamental importance in several large-scale monitoring programs such as the U.S. National Status and Trends program (Wade et al., 1998). By analogy, other suspension feeders with a

similar capacity to metabolically transform ingested hydrocarbons would be alternate candidates for hydrocarbon source identification studies, for example, oysters and clams (Bender et al., 1988) or freshwater mussels.

The time scale of hydrocarbon accumulation and depuration by mussels is generally similar when exposed to oil dispersions or to dissolved hydrocarbons. Typically, 10 to 20 days are required for hydrocarbon concentrations in mussels to approach equilibrium with exposure concentrations (Fossato and Canzonier, 1976; Pruell et al., 1986). Depuration usually follows a two-compartment model, with rapid depuration from one compartment followed by much slower from the other (see review by Meador et al., 1995). Depuration is slower when the exposure route is ingestion of oil droplets compared with dissolved hydrocarbons, suggesting that hydrocarbons absorbed through the gills are less effectively incorporated into somatic tissues than when absorbed through the digestive system. Also, the proportion of hydrocarbons incorporated in the slowly depurating compartment tends to increase with exposure time, probably because of slow exchange between depot lipids and the circulatory system. The depuration half-life of hydrocarbons ingested by mussels is on the order of 3 days for losses from the rapid-depuration compartment (Fossato and Canzonier, 1976). For PAH accumulated from aqueous solutions, depuration half-lives increase with the K_{ow}, are faster at higher temperature, and vary with season and physiological state (Meador et al., 1995). These half-lives typically range from 2 d for naphthalene to as long as 2 weeks for 4-ring PAH.

Vertebrates with substantial capacity to metabolize hydrocarbons can still exhibit hydrocarbon distribution patterns that are little altered from source distributions if the hydrocarbon exposure overwhelms the metabolic capacity of the organism. One of the clearest examples of this occurred following the *Exxon Valdez* oil spill, when contamination of prey across hundreds of square km presented juvenile salmon with a choice between ingesting oiled prey and starvation. Surviving juveniles obviously chose the former, and the PAH distribution of the ingested oil was identical with that of the weathered oil for weeks following the incident, especially in gut samples (Carls et al., 1996). But apart from catastrophic oil spills, the more usual exposure route is through branchial and skin absorption of dissolved hydrocarbons. Even aliphatic hydrocarbons may be absorbed across the intestine when ingested, but fish and other vertebrates readily metabolize this class of hydrocarbons as well, so that only traces of ingested aliphatics persist in the lipid compartment of fish (Cravedi and Tulliez, 1982).

Once incorporated somatically, fish and other vertebrates may transform accumulated PAH into metabolites within hours. Fish metabolize up to 99% of PAH within 24 h of uptake, with most of the metabolites being excreted into the bile (Varanasi et al., 1989). The distribution of PAH among tissues of oiled marine mammals implies metabolic transformation rates that are similarly rapid, with most of the PAH in the liver or in depot lipids of animals that continued to be exposed (e.g., Frost et al., 1994). While it is possible to detect a small proportion of accumulated hydrocarbons in the depot lipids of these animals for considerable periods following exposure, most of the PAH will be present as metabolites, if at all. In fish, biliary PAH metabolites may persist for one to several weeks following cessation of exposure (Krahn et al., 1986). The PAH adducts in DNA are more persistent and may remain detectable for months (Sikka et al., 1991).

The time course of CYP 1A induction and persistence may provide important clues regarding the source of the induction agent. Intraperitoneal injection studies with fish demonstrate that induction is rapid once an inducer has been incorporated into tissues, with elevated CYP 1A levels detectable within hours of injection (e.g., Sleiderink and Boon, 1996) and are paralleled by similar mammalian responses (Renwick et al., 2000). Longer response times of one to a few days are required for field-exposed organisms due to the time required for sufficient contaminant

accumulation to stimulate induction. For readily metabolized inducers such as PAH, the time course of CYP 1A induction typically increases for a few days following the onset of PAH exposure, followed by a decline as the accumulated PAHs are more rapidly metabolized. If the PAH exposure level is steady and chronic, the CYP 1A level declines to a steady-state value determined by a balance between sufficient PAH to maintain induction and the rate of its catabolic transformation (Munkittrick et al., 1995). Once an inducer is no longer available, CYP 1A levels may decline rapidly, with a half-life on the order of a day or less (Sadar and Andersson, 2000). In field studies, CYP 1A induction may be evident for weeks or months because of repeated exposure, or because of release from lipid depots as they are being drawn down (Kennish et al., 1992). But in contrast with PAH, less readily metabolizable inducers such as polychlorinated biphenyls or dioxin may continue to stimulate induction for weeks following a discrete exposure event (van der Weiden et al., 1994; Beyer et al., 1997), so the persistence of CYP 1A following the end of exposure to the inducing medium provides an indication of the chemical class of the inducing agent responsible.

Environmental and other factors can modify the time course of CYP 1A induction by PAH, which should be considered when interpreting these induction patterns (reviewed by Whyte et al., 2000). Even among vertebrates, the strength of the induction may vary considerably among species, within species of differing sizes and especially with life stage and reproductive condition, with lower induction occurring in early life stages and in reproductively active females (Lindström-Seppä and Stegeman, 1995). Lower temperatures retard induction, as does poor nutritional status of the organism. Nonetheless, the sensitivity of the response to PAH exposure is sufficiently robust that most vertebrates will exhibit detectable induction following a sufficiently large exposure, with the advantage that the CYP 1A response may well remain evident when PAHs are not, due to metabolic transformation. For example, rockfish collected after the *Braer* oil spill had CYP 1A levels nearly an order of magnitude above constitutive levels for months after the incident when PAHs were undetectable, and the CYP 1A response increased with proximity to the accident site (George et al., 1995). These results clearly demonstrate protracted exposure of these fish to bioavailable PAH in the subtidal zone of the spill-affected area.

In summary, the spatial and temporal patterns of hydrocarbon accumulation by biota, or of biological biomarkers of exposure to hydrocarbons, may provide direct or supporting evidence regarding the sources responsible. When it can be shown that the biota sampled have a negligible effect on the composition of the hydrocarbons accumulated, these data may be sufficient to identify the source unambiguously. Biological biomarker responses may provide supporting evidence that may be used to reduce the number of plausible sources, as may biomarkers that are based on higher levels of biochemical and physiological integration, through that of the individual organism. The following section summarizes these higher-level biological responses of organisms to hydrocarbon exposure, which may be used in a similar manner with the biological biomarkers as evidence for determining the source(s) responsible.

12.4 Modes of Toxic Action of Accumulated Hydrocarbons

A number of organism-level responses to hydrocarbon exposure have been described, and some of these have a specificity approaching that of the biological biomarkers discussed in Section 12.3. Exposure to hydrocarbons, and especially to PAH, causes a variety of organism-level responses, depending on the mode of exposure, the species, the developmental status of the target, and the time scale of observation. Perhaps the most widely applied, yet least sensitive, of these exposure indicators is cumulative mortality induced through narcosis. Oil pollution and PAH had once been regarded as minimally toxic based on the relatively high exposure concentrations

required to kill test organisms within a few days. But within the last decade, the ecological consequences of reduced fitness have become more fully appreciated, with the recognition that even a small reduction in the ability to acquire prey or to avoid predators in the wild may lead to mortality prior to the first reproductive opportunity, and that these effects have the potential to harm a population as certainly as mortality from acute toxicity (Rose et al., 2003). Such eventual adverse effects may result from impaired development, growth inhibition by ingested hydrocarbons, immune suppression, or carcinogenesis. As with the biological biomarkers, the spatial and temporal patterns of these responses may indicate particular hydrocarbon sources of exposure.

The aqueous concentrations of hydrocarbons that are necessary to kill aquatic biota through narcosis are so high that such mortality is not usually observed in conjunction with exposure to oil pollution in the field. Narcosis-induced mortality arises when nonpolar contaminants such as hydrocarbons accumulate in the neural membrane lipids, inhibiting nerve transmission, and causing death through asphyxiation or heart failure. The PAH concentrations required to cause lethal narcosis are on the order of 5×10^{-5} mole/g lipid, corresponding to about 2.5×10^{-6} mole/g wet weight of the whole organism if it is 5% lipid (DiToro et al., 2000). Assuming again that the K_{ow} is a reasonable approximation of the equilibrium distribution of a hydrocarbon in the lipid and the aqueous exposure water, a lethal exposure concentration may be expressed as $(5 \times 10^{-2}/K_{ow})$ moles/L.

Comparison of the ratio $(5 \times 10^{-2}/K_{ow})$ moles/L with the expected concentration of a hydrocarbon in equilibrium with the oil phase ($\approx C_{so}/(K_{ow} + Q)$; see Section 12.2) shows that lethal narcosis would only occur when the oil phase contains a high proportion of the most soluble hydrocarbons (i.e., BTEX) in contact with a limited amount of the aqueous phase. For example, an oil phase containing 10% benzene (about 0.13 mole/L, K_{ow} of 135) would produce a lethal aqueous concentration only at water-to-oil volume ratios smaller than about $Q = 200$. Most oil-derived products do not contain such high concentrations of BTEX and are not confined with such limited quantities of water following environmental release. Consequently, narcosis-induced mortality is usually associated with products with high BTEX contents such as gasoline and some diesel oils, when energetically mixed in confined waterways. Because of the high vapor pressures of the BTEX compounds, the potential for narcosis-induced mortalities is also ephemeral, as these hydrocarbons readily evaporate on time scales of hours to a day or so.

Chronic exposure to PAH-contaminated sediments can elicit a complex of cancerous tumors in fish. A particularly clear linkage was established between liver neoplasms of brown bullhead catfish (*Ameiurus nebulosus*) and PAH associated with a coking facility on the Black River of Ohio (Baumann and Harshbarger, 1995). Sediment PAH and liver cancers in the catfish declined simultaneously following closure of the facility in 1982. Disturbance of the sediments from dredging in 1990 led to an increase of tumors during the following 2 to 3 years, and the population age structure of the affected fish was consistent with exposure during 1990 (Baumann and Harshbarger, 1998). These results corroborated extensive studies of Puget Sound, Washington, that implicate exposure to PAH in sediments as contributing agents to carcinogenesis in fish there (Myers et al., 1990).

More subtle but insidious biological manifestations of exposure to hydrocarbons are the effects of PAH on the early developmental stages of fish. Research motivated by field observations following the *Exxon Valdez* oil spill, and by concerns associated with pulp mill effluents, has led to a number of recent studies implicating the role of the more environmentally persistent 3- and 4-ring PAHs, which elicit a suite of symptoms similar to those produced by dioxin exposure (Incardona et al., 2004, 2005). This syndrome, sometimes denoted as "blue-sac disease," can include pericardial and yolk sac edema, hemorrhages,

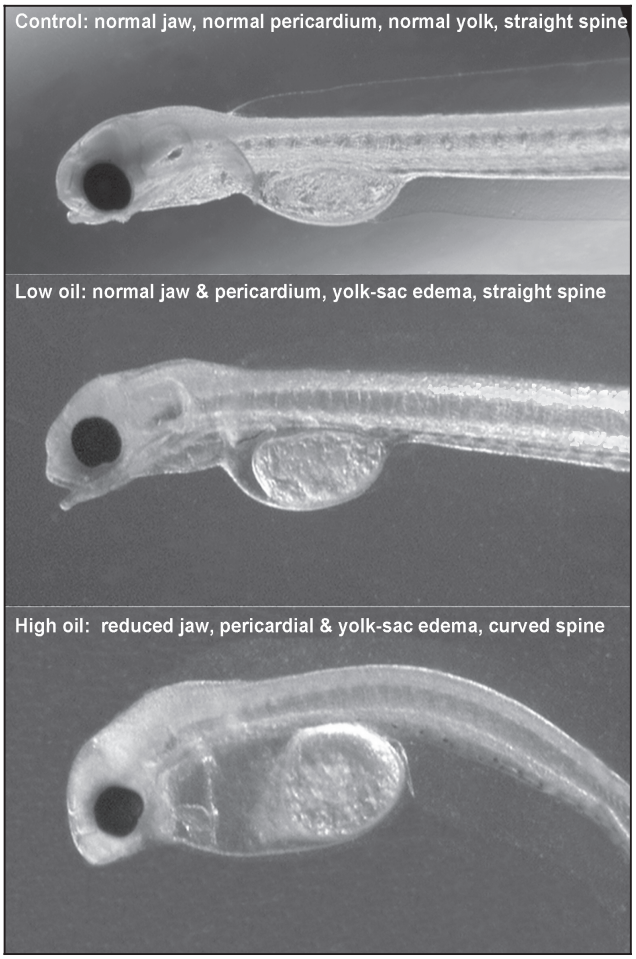

Figure 12-7 Some effects of oil exposure in herring larvae (photos courtesy of NOAA).

craniofacial and spinal malformations, and mortality (Billiard et al., 1999; Carls et al., 1999; Heintz et al., 1999; Couillard, 2002) and may occur following embryonic exposures to PAH concentrations in the range of 1–30 µg/L. Its severity is dose-response-related, as shown in Fig. 12-7. Unlike narcosis-mediated mortality, these concentrations may be readily attained following environmental releases of oil products. For example, the combined concentration of parent and alkyl-substituted phenanthrenes and dibenzothiophenes, both of which are implicated in blue-sac disease syndrome, in Alaska North Slope (ANS) crude oil is about 3×10^6 µg/L (Wang et al., 2003), and their K_{ow} is on the order of 10^5. The expression $C_w = C_{so}/(K_{ow} + Q)$ implies that water equilibrated with ANS oil could attain combined concentrations of these PAH of ~10 µg/L at water-to-oil volume ratios (Q) on the order of 2×10^5. Hence, 1 L of ANS oil could contaminate 200 m^3 of water with this concentration. This suggests that the appearance of this syndrome may be a sensitive indicator of PAH exposure, either in natural populations sampled from their natal habitat or in captive organisms deliberately exposed to assess field conditions (Couillard, 2002), provided other potential inducers such as dioxins can be discounted.

Exposure to hydrocarbons may cause other less observable adverse effects on biota. Ingestion of aliphatic hydrocarbons may cause marked inhibition of growth in fish (Luquet

et al., 1984), and although the metabolic mechanism underlying this is not clear, such growth inhibition has been documented in fish that have ingested oil following the *Exxon Valdez* oil spill (Carls et al., 1996). Some of the PAHs in oil products are also phototoxic, meaning that they may catalyze production of reactive oxygen species inside cells that accumulate them when irradiated by the ultraviolet component of sunlight (Pelletier et al., 1997).

These macroscopic responses of biota may provide essential clues of exposure to hydrocarbons when their involvement is not already confirmed by other evidence, such as proximity to an accidental oil spill. For example, the appearance of tumors and deformities in aquatic biota might serve as the first indication that the ambient water may be receiving a cryptic input of hydrocarbons, prompting more definitive sampling. Examination of biological biomarkers in exposed biota may then provide indications of the route of exposure and perhaps its spatial location. From this perspective, these macroindicators may serve as the initial indicators of potential hydrocarbon exposure, prompting use of more involved but reliable environmental sampling methods.

Many of the advances in our understanding of how hydrocarbons may permeate the biotic components of natural ecosystems stemmed from studies of the *Exxon Valdez* oil spill, easily the most intensively studied oil pollution event in history. The following section summarizes the most noteworthy findings of that event in regard to the biological responses and indicators of exposure to the oil, as an example of the processes and responses described above.

12.5 Case Study: The *Exxon Valdez* Oil Spill

The opportunity provided by the *Exxon Valdez* oil spill to study the environmental effects of oil pollution was unique, and not likely ever to be repeated. As a large perturbation in an otherwise mostly pristine region, its effects were rarely confounded by other sources of pollution. Although remote, the impacted area within Prince William Sound, Alaska, is at the edge of accessibility by road, three ports, and commercial air service, and is within 100 km of a large population center, facilitating long-term scientific studies. Finally, unprecedented funds were made available for such studies, permitting examination of questions that otherwise would have been neglected. These unique circumstances have led to a particularly clear illustration of the biological responses to the spilled oil as it made its way through the ecosystem. In retrospect, these responses show how hydrocarbons accumulated by biota — and the biotic responses to those accumulations — are related to the environmental dispersion of the oil.

To appreciate the biotic responses to the oil, it will be helpful to summarize the dispersion of the oil in the environment and the physical factors underlying the dispersive processes. The overall fate of the oil was largely determined by weather events during the first few weeks. Oil discharge began shortly after midnight on Friday, March 24, 1989, during calm conditions at seawater temperatures of about 5°C. Wind speeds remained variable and below about 5 m/sec for the next three days, allowing the oil slick to remain a compact and roughly circular pool that spread rapidly at rates approaching 2000 m^2/sec to an area of nearly 400 km^2, with thicknesses that decreased from generally less than 1 mm after the first few hours of discharge to perhaps 0.1 mm by Sunday. The rapid increase in the surface area of the oil accelerated evaporation of the most volatile components, leading to losses of ~15% by weight, including nearly all of the BTEX, and of the saturated hydrocarbons with vapor pressures greater than that of dodecane (Payne et al., 1991).

An intense storm with winds at speeds of up to 35 m/sec disrupted the slick and drove it toward beaches during the next 3 days. The first landfall of the slick occurred with breaking waves of 1–3 m. Initially, the main effects of the high seas associated with the storm were to disperse oil as small droplets into the water column and to promote water incorporation into the oil. Oil droplet dispersion occurred at

least to depths of 25 m (Short and Harris, 1996a), and probably considerably deeper because the density of the water column varies little with depth near the end of winter in Prince William Sound (Vaughan et al., 2001). The large surface-to-volume ratio associated with these small oil droplets promoted dissolution of the more readily soluble oil components into the seawater column, leading to concentrations of PAH (mainly dissolved) that totaled a few parts per billion weeks after the storm had passed (Neff and Stubblefield, 1995; Short and Harris, 1996b).

The high storm winds caused slick thicknesses to increase adjacent to the initially impacted beaches, and the breaking waves caused substantial accumulations of oil to be stranded on beaches during falling tides, often blanketing the ~4-m vertical tidal excursion with oil. On porous beaches, this process allowed pools of stranded oil to percolate into subsurface sediments as the water table lowered during the outgoing tide. Even the fine-grained sediments of these beaches have high permeabilities (Bragg and Yang, 1995), so the intertidal water table follows the tide level closely, permitting the more viscous oil hours to seep into the underlying sediments, to depths of 1 m in some locations (Neff et al., 1995). Subsequent rising tides failed to completely remove this subsurface oil because the increased oil viscosity inhibited displacement from finer-grained sediments. This initial percolation of oil into subsurface sediments set the stage for long-term persistence there.

The dispersion and environmental persistence of the spilled oil were clearly reflected in the associated biota. Mussels were the most extensively sampled sentinel organisms, and when elevated, the PAH distributions found in them were nearly always consistent with the weathering patterns that characterized the whole oil (Short and Heintz, 1997), including association with phytane (e.g., Short and Harris, 1996a). Corroborating patterns were also evident in other suspension-feeders, such as clams (Short and Heintz, 1997), and in the guts of juvenile salmonids and other animals that ingested oiled prey (Bence and Burns, 1995; Carls et al., 1996). Both the spatial and temporal patterns of hydrocarbon accumulation by biota showed clear relationships with proximity to beaches that were heavily oiled (e.g., Short and Harris, 1996a).

Exposure of biota to dissolved hydrocarbons from the spilled oil was less clear, because of the large capacity for dilution and because of difficulties distinguishing PAH concentration patterns indicative of dissolved species from those characteristic of diesel oil. Enormous effort was expended during attempts to clean oiled beaches and the sea surface, which involved a host of support vessels that were potential ancillary sources of hydrocarbon contamination. Diesel oil from these vessels, as well as from scientific support vessels, almost certainly contaminated some of the samples collected (Bence and Burns, 1995). When available, data for associated aliphatic hydrocarbons such as phytane may be used to differentiate between exposure to diesel oil vs. the water-soluble components of crude oil (as in the decision-tree approach of Bence and Burns, 1995).

Longer term, oil in a semi-liquid state remained sequestered within the most heavily oiled beaches throughout the 1990s, providing a reservoir that could contaminate organisms that forage by disturbing these sediments (Short et al., 2004). Studies of sea ducks and otters consistently found biochemical evidence of exposure to hydrocarbons in the form of elevated CYP 1A levels in animals that increased with proximity to the lingering oil and that steadily declined with time as the years progressed (Trust et al., 2000; Esler et al., 2002; Bodkin et al., 2002), reflecting either the gradual dissipation of the lingering oil on the beaches, or possibly learned avoidance behavior.

A more subtle PAH-exposure pathway was revealed by CYP 1A measurements on wild pink salmon embryos. Salmon spawning streams usually escaped direct oiling, even on otherwise heavily oiled beaches, because the freshwater stream flow diverted oil away from them. Hence, while PAH concentrations in sediments of these streams were usually well

below 1000 ng/g (Brannon et al., 1995), they were higher by factors of 10–1000 in sediments adjacent to the stream channels (Murphy et al., 1999). Induction of CYP 1A was found in 13 of the 16 samples of pink salmon eggs incubating within gravels of four streams where the adjacent beaches had been heavily oiled. In contrast, no induction was noted in any of the seven samples from five reference streams on unoiled beaches. A field study subsequently confirmed that groundwater movement following hydraulic gradients within these beaches as the tides fall would readily transport PAH dissolved from adjacent oil to the subsurface stream flow where the eggs were incubating (Carls et al., 2003). This unexpected exposure pathway would likely not have been detected without the indications provided by the CYP 1A measurements of the developing embryos. As with the sea duck and otter studies, these spatial and temporal patterns of biochemical responses to oil exposure demonstrate the power of these responses to implicate pollution sources. In fact, it was the unexpected appearance of these responses in biota during the 1990s that motivated the study to evaluate the long-term persistence of the oil on the beaches in 2001 (Short et al., 2004).

12.6 Summary

Whether direct or indirect, assessments of hydrocarbons in biota may provide evidence that is crucial to identifying pollution sources. Biological samples are often collected and analyzed for hydrocarbons, or for evidence of exposure to hydrocarbons, as part of damage assessments associated with pollution-monitoring efforts, and useful information regarding pollution sources may often be gained from relatively little additional chemical or data analysis. When the hydrocarbon distribution pattern is not substantially altered along the exposure pathway or by the biota through biochemical transformation, then these patterns may provide direct evidence of the source, comparable with environmental samples of the released hydrocarbon source material. But even when considerable alteration occurs, spatial and temporal patterns of biological biomarkers indicative of hydrocarbon exposure may still furnish evidence regarding potential sources. The most widely used biological biomarker has been associated with the phase I metabolic transformation pathway involving induction of the CYP 1A detoxification enzyme (e.g., the EROD assay), but other biomarkers associated with phase II condensation reactions (e.g., bile analytes of anthropogenic PAH), as well as toxic responses at the organismal level of affected biota, have also proved useful. The source information available from such approaches and analyses has the potential to be pivotal when evaluating potential sources of environmental hydrocarbon pollution.

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