10

Biomarkers

The term biomarker has been gaining acceptance in recent years, albeit with some inconsistency in definition. Here we define a biomarker as any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status. Thus biochemical, physiological, histological, morphological, and behavioral measurements may be considered biomarkers. Table 10.1 illustrates examples of biomarkers at different organizational levels. Biological responses at higher organizational levels—population, community, and ecosystem—are considered bioindicators. Notwithstanding the importance of changes at these higher levels (Chapters 12, 15, and 16), they are too general to be considered specific biomarkers.

The relationship between biomarkers and bioindicators based on specificity and ecological relevance is shown in Figure 10.1. In general, it is difficult to relate biochemical changes to ecological changes (although eggshell thinning caused by p,p´-DDE and imposex in dog whelks caused by TBT, discussed in Chapter 16, show that a physiological change can be related to a massive population change). It is also difficult to relate ecological changes to specific chemical causes.

10.1 Classification of Biomarkers

A number of classifications of biomarkers have been proposed. The most widely used is division into biomarkers of exposure and biomarkers of effect. Biomarkers of exposure indicate exposure of an organism to chemicals but do not indicate the degree of adverse effect the change causes. Biomarkers of effect, or more correctly toxic effect (because all biomarkers by definition show effects) demonstrate adverse effects on organisms.

Figure 10.2 depicts a biomarker approach based on changes in physiological parameters. The change in the health status of an individual with increasing exposure to a chemical is shown by a smooth curve running from healthy through reversible to irreversible changes leading to death. The important transition points along the way are: (i) first stress (h), i.e., physiology is no longer normal and the organism, although stressed, is able to compensate for this stress; (ii) the organism is no longer able to compensate (c) but the changes are still reversible and removal of the stress enables the organism to recover; and (iii) the point r beyond which the changes are irreversible and death ensues. The second part of Figure 10.2 shows the responses of five biomarkers used to measure health status (Depledge et al., 1993; Depledge, 1994).

TABLE 10.1

Biomarkers at Different Organizational Levels

Organizational Level	Example of Biomarker
Binding to receptor	TCDD binding to Ah receptor
	Nonylphenols binding to estrogen receptor
Biochemical response	Induction of Cytochrome P_{450}IA
	Inhibition of ACh-ase
	Vitellogenin formation
Physiological alteration	Eggshell thinning
	Feminization of embryos
Effect on individual	Behavioral changes
	Scope for growth

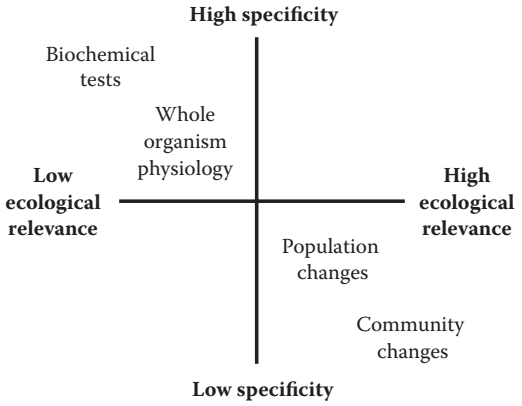

FIGURE 10.1
Specificity and ecological relevance of biochemical effects measurements. (*Source:* Addison, F. (1996). *Environmental Reviews* 4, 225–237. With permission.)

10.2 Specificity of Biomarkers

Biomarkers range from those that are highly specific—an enzyme of the heme pathway known as aminolevulinic acid dehydratase (ALAD) is inhibited only by lead—to those that are nonspecific. Effects on the immune system can be caused by a wide variety of pollutants. Table 10.2 lists biomarkers by degree of specificity.

Both highly specific and highly nonspecific biomarkers have value in hazard assessment. Taking blood samples of waterfowl and measuring ALAD activity can determine the percentage of the waterfowl at risk from lead poisoning without further measurements. However, determination of ALAD does not indicate what other pollutants may be present.

Inhibition of AChE can be used to provide legal proof of death by organophosphorous and carbamate pesticides (Hill and Fleming, 1982), and such inhibition is considered specific to these classes of chemicals. However, in recent years, evidence indicates that the inhibition of AChE is not caused solely by OPs and carbamates. Payne et al. (1996) found depression of AChE activity as high as 50% in fish in Newfoundland remote

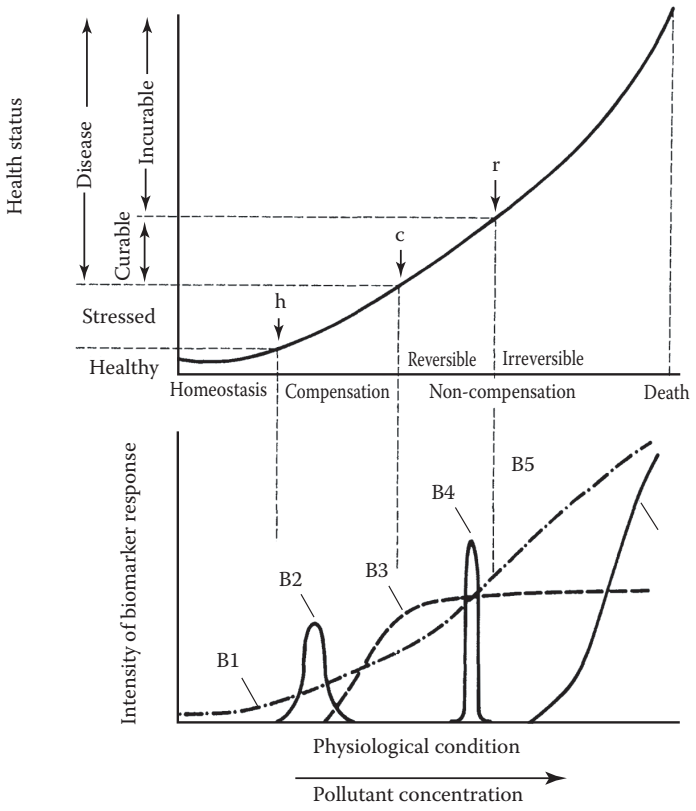

FIGURE 10.2
Relationship of exposure to pollutants, health status, and biomarker responses. Upper curve shows the progression of the health status of an individual as exposure to pollutant increases. *h* = Point at which departure from normal homeostatic response range is initiated; c = limit at which compensatory responses can prevent development of overt disease; r = limit beyond which the pathological damage is irreversible by repair mechanisms. The lower graph shows responses of five hypothetical biomarkers used to assess the health of the individual. (*Source:* Depledge. M.H. et al. (1993). In *Biomarkers: Research and Application in the Assessment of Environmental Health*. Springer-Verlag. With permission.)

from pesticide use and considered that a complex mixture of pollutants may have been involved. Studies by Guilhermino et al. (1998) noted that both detergents and metals can inhibit the activity of AChE and suggest that use of this enzyme as a biomarker could be extended.

The induction of monooxygenase is caused by a wide variety of chemicals and is often a sensitive indicator of pollutants (Besselink et al., 1997). Thus it is a useful indication that organisms are affected by pollutants, although it seldom reveals a specific cause. An exception is the induction of cytochrome P_{450}1A1 and 1A2 by planar compounds such as dioxins and coplanar PCBs. Here, the degree of induction of the enzyme can give a measure of Ah receptor-mediated toxicity that may be caused by these pollutants. See Walker (2009) for further discussion. The induction of other P_{450}s that have less substrate specificity can provide the basis for useful biomarker assays of exposure without providing much evidence for identifying pollutants causing the response.

TABLE 10.2

Biomarkers Listed by Decreasing Specificities to Pollutants

Biomarker	Pollutant	Comments and References
ALAD inhibition	Lead	Sufficiently reliable to replace chemical analysis (Wigfield et al., 1986)
Metallothionein induction	Cadmium	More difficult to measure than cadmium levels (Hamer, 1986)
Eggshell thinning	DDT, DDE, dicofol	Degree of eggshell thinning is easily measured (Ratcliffe, 1967)
AChE inhibition	OPs, carbamates	Easier and more reliable than chemical analysis (Fairbrother et al., 1991)
Anticoagulant clotting proteins	Rodenticides	Measurements similar in complexity to chemical analysis (Huckle et al., 1989)
Monooxygenase induction	OCs, PAHs	Dioxin equivalent more easily measured than chemical analysis (Murk et al., 1997)
Porphyrin profiles	Several OCs	Separation by high-performance liquid chromatography well developed (Kennedy and James, 1993)
Retinol profiles	OCs	Demonstrates exposure to specific chemicals (Shugart, 1994); considerable natural variations; ratios more reliable than absolute values (Spear et al., 1986)
DNA and hemoglobin adducts	Largely PAHs	Several tests available; complicated by repair mechanisms
Vitellogenin induction	Estrogenic chemicals	Induction in male fish is sensitive indicator of estrogenic chemicals (Harries et al., 1997)
Other serum enzymes	Metals, OCs, PAHs	Several enzyme systems have been studied (Fairbrother, 1994)
Stress proteins	Metals, OCs	Wide range of stress proteins have been studied (Sanders, 1993)
Immune responses	Metals, OCs, PAHs	Many tests available (Wong et al., 1992)

10.3 Relationship of Biomarkers to Adverse Effects

The ability to relate a degree of change of a biological response to the harm it causes is useful, primarily for defending the cost of a proposed remedial action. Tabled 10.3 lists the same biomarkers shown in Table 10.2 in the order of the adverse effects they measure. A quick examination of the two tables will reveal differences in ranking order.

The first biomarker in Table 10.3 is eggshell thinning. It is possible to define a critical degree of eggshell thinning; it has been found for a variety of species that eggshell thinning in excess of 16 to 18% is associated with population declines. This phenomenon is discussed in more detail in Chapter 16.

The fact that the relationship between the biomarker response and an adverse effect is not clear does not invalidate the use of the biomarker. First, it demonstrates that the organism has been sufficiently exposed to a pollutant or pollutants to undergo a physiological change. In cases such as the induction of metallothionein, the change is protective (Chapter 7); a knowledge of how much of the possible protective mechanism

TABLE 10.3

Biomarkers Listed by Decreasing Specificities of Adverse Effects

Biomarker	Organizational Level	Comments and References
Eggshell thinning	Intact animal—population	Wide species variation in sensitivity; related to reproductive success (Peakall, 1993)
Inhibition of AChE	Organ—intact animal	Degree of inhibition related to mortality and sublethal effects (Grue et al., 1991)
Inhibition of ALAD	Organ—intact animal	Degree of inhibition related to mortality (Scheuhammer, 1989)
Clotting proteins	Intact animal—population	Related to mortality; risk assessed from blood protein levels (Hegdal and Blaskiewicz, 1984)
Induction of monooxygenases	Organ—population	Analysis of dioxin equivalents related to reproductive success; induction of P_{450} enzymes related to specific chemicals (Bosveld and van den Berg, 1994)
Depression of plasma retinol and thyroxine	Organ	Binding to specific protein; relation to adverse effects tenuous (Brouwer and van den Berg, 1986)
DNA integrity	Organ	DNA damage indicates serious harm; relationship to effects often tenuous (Everaarts et al., 1998)
Immune responses	Organ	Proper functioning critical to health, but system has considerable reserve (Richter et al., 1994)
DNA and hemoglobin adducts	Organ	Good monitor of exposure, especially for PAHs; relation to effects is tenuous (Varanasi et al., 1989)
Other enzymes	Organ	Relationship to effects not clear (Fairbrother, 1994)
Porphyrin profiles	Organ	Levels in environmental samples well below those causing adverse effects (Fox et al., 1988)
Induction of vitellogenin	Organ—intact animal	Clear link between induction of vitellogenin and presence of estrogenic chemicals; biological significance is speculative (Arcand-Hoy and Benson, 1998)
Induction of metallothionein	Organ	Protective mechanism, not related to mechanism of toxicity (Hamer, 1986)
Stress proteins	Organ	Difficult to separate effects from nonchemical stresses (Pyza et al., 1997)

has already been induced is valuable to assess the risk to individuals. Second, in the case of vital systems, it is an indication that further investigations should be undertaken. For example, few would take damage to the integrity of DNA lightly, even though in many cases the damage is repaired and no adverse effects occur (Chapter 7). In other cases, such as changes in porphyrin levels, it is clear that the levels are much lower than those shown to cause harm. Nevertheless, these biomarkers can be used to demonstrate exposure. The use of these various types of biomarkers in hazard assessment is considered later in this chapter.

10.4 Specific Biomarkers

Some of the biomarkers listed in Tables 10.2 and 10.3 are covered elsewhere in this book. Specifically, eggshell thinning is discussed in Chapter 16, and some information about the inhibition of AChE and induction of monooxygenases appears in Chapter 7. The next sections discuss some of the available biomarkers. More complete coverage is given in the books on biomarkers cited on the reading list at the end of this chapter.

10.4.1 Inhibition of Esterases

From the point of view of ecotoxicology, AChE is particularly useful because it represents the site of action, and its degree of inhibition relates to toxic effects. Butyrylcholinesterase (BuChE) is sometimes studied in parallel with AChE, but its physiological role is unknown and its degree of inhibition is not simply related to toxic effect. The study of neuropathy targets esterase, the interaction of which with organophosphorous compounds (OPs) can lead to organophosphorous compound-induced delayed neurotoxicity, has been confined to laboratory studies.

The mode of action is well established and has been considered in some detail in Chapter 7. Two classes of compounds, the OPs and carbamates, inhibit AChE, causing an accumulation of acetylcholine at the nerve synapses and disruption of nerve function that produces obvious effects: tremors, motor dysfunction, and death. The assay for AChE is more straightforward, quicker, and cheaper than chemical analysis for OPs or carbamates. The degree of inhibition of AChE has been related to the symptoms observed.

With vertebrates, the inhibition of brain AChE has often been used to establish that death has been caused by OP or carbamate pesticides (Mineau, 1991). Under ideal conditions, inhibition in the range of 50% to 80% can be taken as proof of mortality from the pesticide (Hill and Fleming, 1982). In practice, the degree of denaturation is often unknown and adequate controls are often difficult to obtain. Also, inhibition by carbamates is readily reversible (cf. OPs) and can be quickly lost after death. However, the measurements of OPs and carbamates are made more difficult because they are rapidly metabolized and eliminated; in fact, chemical analysis for residue levels has not been widely used for diagnosing poisoning by these compounds. The use of AChE inhibition for diagnosing damage caused by pesticides as a consequence of forest spraying in eastern Canada is discussed in Chapter 16.

The usual wild vertebrate organ studied is the brain—the principal site of action of OPs and carbamates. Although using inhibition of blood AChE or BuChE would be more acceptable, the relationship to inhibition of brain AChE is complex. Studies have shown that variability of esterase activity is much greater with plasma than with brain and that the recovery of plasma AChE activity is much more rapid than recovery of brain AChE. Also, plasma AChE does not represent a site of action of OPs and carbamates, and there is no simple relationship between degree of inhibition and toxic effect. Thus diagnosis based on plasma AChE activity is difficult.

10.4.2 The Induction of Monooxygenases

The heme-containing enzymes known as cytochromes P_{450} are major components of the defenses of organisms against toxic chemicals in their environment. Originally evolved, perhaps as long as 2,000 million years ago, to handle naturally occurring toxic compounds

(Nebert and Gonzalez, 1987), they now play an important role in the detoxification of man-made chemicals.

The monooxygenase system is a coupled electron transport system composed of two enzymes—a cytochrome and a flavoprotein (NADPH-cytochrome reductase). The system is found in the endoplasmic reticulum of most organs, but the activity is far greater in the liver than in most other tissues (Chapter 5). Recent work has shown the complexity of the system. One review identifies more than 750 isoforms belonging to 74 different gene families (Nelson, 1998). Cytochrome P_{450} I-dependent reactions include N-oxidation and S-oxidation; widely studied enzyme activities include ethoxyresorufin O-deethylase (EROD), benzo(a) pyrene hydroxylase (BaPH), and aryl hydrocarbon hydroxylase (AAH). Cytochrome P_{450} II-dependent oxidations include aromatic hydroxylation, acyclic hydroxylation, dealkylation, and deamination. Monooxygenases are induced by a wide variety of compounds (Section 7.2). Inducers of environmental interest include some organochlorine, organophosphorous, and pyrethroid insecticides, polycyclic aromatic hydrocarbons (PAHs), PCBs, and TCDDs.

Using the induction of monooxygenase activity in fish as a monitor of pollution of the marine environment by oil was proposed in the mid 1970s. Since then, a wide variety of studies has been published, ranging from the induction of AHH in fish near the Los Angeles sewage outlets to EROD induction by pulp mill effluent in Sweden. Induction of monooxygenases by paper mill effluent has proven one of the most sensitive biomarkers for tracking this type of pollution.

Monooxygenase activity is shown by a wide range of species (see Chapters 5 and 7), and some studies on fish-eating birds in the Great Lakes are detailed in Chapter 16. The value of monooxygenases for biological monitoring has been clearly demonstrated in the case of hydrocarbon pollution in fish and aquatic invertebrates and for both PAH and OC contamination in a wide range of organisms. As the response is caused by a very wide variety of chemicals, the system is capable of detecting exposures sufficiently high to cause biological responses to many xenobiotics. Conversely, it has only limited value for identifying causative agent(s) but can be used to delimit an area that may later warrant more detailed investigations.

From a practical view, the considerable variation within a specific population means that the sample size usually must be fairly large. If a system is induced by a large variety of natural compounds and xenobiotics and is affected by a wide variety of other parameters—temperature, diet, etc.—great care must be taken to ensure there are reliable control levels.

10.4.3 Studies of Genetic Materials

The fundamental role of DNA in the reproductive process is well known and will not be discussed further. The end points used to assess the damage to DNA by environmental pollutants are specific genotoxic effects, especially increased carcinogenesis, rather than effects on the reproductive process.

The sequence of events between the first interaction of a xenobiotic with DNA and consequent mutation may be divided into four broad categories. The first stage is the formation of adducts. At the next stage, secondary modifications of DNA such as strand breakage or an increase in the rate of DNA repair occur. The third stage is reached when the structural perturbations to the DNA become fixed. At this stage, affected cells often show altered functions. One of the most common assays to measure chromosomal aberrations is sister chromatid exchange. Finally, when cells divide, damage caused by toxic chemicals can lead to the creation of mutant DNA and consequent alterations in gene function.

The covalent binding of reactive metabolites of environmental pollutants to DNA—adduct formation—is a clear demonstration of exposure to these agents and an indication of possible adverse effects. The relationships of environmental levels, degrees of adduct formation, and ultimate effects are complex. For example, although a direct relationship between the extent of cigarette smoking and the number of DNA–BaP adducts has been clearly shown, the relationship between DNA–BaP adducts and the onset of lung cancer is less well defined. In the field of wildlife toxicology, the establishment of the sequence of events from the initial DNA lesion to harm is even more difficult. Nevertheless, it is reasonable to conclude that the reactions of chemicals with DNA cause harmful consequences such as tumor formation.

Three approaches have been used to study DNA damage caused by genotoxins and the formation of DNA adducts, after exposure to pollutants:

1. Radioactive postlabeling (usually with ^{32}P), leading to the separation of a range of adducts by two-dimensional thin-layer chromatography
2. Techniques to identify specific adducts, including fluorescence spectrometry, chromatographic techniques, and enzyme-linked immunosorbent assay (ELISA)—techniques that are sensitive if properly used and can detect one adduct among 10^8 normal nucleotides
3. Random amplified polymorphic DNA (RAPD) assays to detect alterations to DNA caused by chemicals

The three techniques yield different data. Radioactive postlabeling reveals the degree of total covalent binding. The techniques for identifying specific adducts provide information about the degrees of binding for a few specific compounds. RAPD gives evidence of genotoxin- induced DNA damage and mutations (Atienzar and Jha, 2004).

Monitoring of adduct formation provides one of the best means of detecting exposure to polycyclic aromatic hydrocarbons (PAHs). The stability of DNA and hemoglobin adducts formed by this class of compounds means that evidence of exposure to them remains after the compounds are cleared from the body. The ability to study adduct formation by hemoglobin means that nondestructive testing is possible.

In studies at Puget Sound, Washington, fish and sediments were sampled. The levels of PAHs in sediment and gut were determined, and the extent of DNA–xenobiotic adducts in the liver measured by ^{32}P labeling. Additionally, concentrations of PCBs and the degree of induction of MFOs were determined. The various indices enabled workers to discriminate sites that exhibited considerable differences in chemical contamination by both PCBs and PAHs (Stein et al., 1992).

DNA fingerprinting using the polymerase chain reaction has also been used as a biomarker for the detection of genotoxic effects of environmental chemicals (Savva, 1998; Atienzar et al., 1999). The differences between control and experimental fingerprints are thought to be caused by DNA adducts. In studies of bivalves in the lagoon of Venice, good correlation was found between DNA fingerprinting and the degree of strand breakage measured by the alkaline unwinding assay (Castellini et al., 1996).

Breakage in chromosomes can be examined directly under a microscope or by the alkaline unwinding assay (Peakall, 1992). This technique is based on the DNA strand separation that takes place where there are breaks. The amount of double-stranded DNA remaining after alkaline unwinding is inversely proportional to the number of strand breaks, provided that renaturation of the DNA is prevented.

Chromosome breaks in the gills of mud minnows were used to study pollution of the Rhine. Also, increased chromosomal aberrations were found in rodents collected from areas near a petrochemical waste disposal site. Although damage to chromosomes can lead to serious effects, it must be remembered that repair mechanisms are capable of preventing them.

Sister chromatid exchange (SCE) is the reciprocal interchange of DNA during the replication of chromosomal DNA. Chromosomes of cells that have gone through one DNA replication in the presence of labeled thymidine or the nucleic acid analogue 5-bromodeoxyuridine and then replicated again in the absence of the label are generally labeled in only one of the chromatids. The label is exchanged from one chromatid to the other. The SCEs can be easily visualized using a light microscope or differential staining.

Chromosomes that have undergone SCE should not be regarded as damaged in the conventional sense because they are morphologically intact. Nevertheless, SCE occurs at sites of mutational events including chromatid breakage. Good correlations have been observed between the number of induced SCEs per cell against the dosage of x-rays and the concentration of a number of chemicals known to cause chromosomal aberrations.

A relationship between SCE level (Nayak and Petras, 1985) and distance from an industrial complex was demonstrated in wild mice in Ontario, Canada. A variety of chromosomal aberrations were found in cotton rats living near hazardous waste dumps in the US Fish exposed to water from the Rhine showed marked increases of SCE levels (van der Gaag et al., 1983).

Flow cytometry can detect chromosomal aberrations in a large number of cells rapidly and accurately. It has been shown to detect mutagenic and clastogenic effects in a wide variety of species (Bickham et al., 1998; Whittier and McBee, 1999).

A number of changes in genetic materials can be used to monitor for pollution by specific assays. Monitoring has been carried out on the incidence of tumors in fish. Fish are frequently sampled in considerable numbers and many of their tumors are visible externally. Such data could not be readily collected from other classes of organisms (muskrats from trappers or ducks from hunters) even when large numbers of samples are available because of the cost of dissection.

In the North American Great Lakes, surveys to determine the incidence of tumors were conducted as part of a surveillance program. The levels of occurrence of tumors in brown bullheads (*Ictalurus nebulosis*) and white suckers (*Catostomus commersoni*) were highest in the most polluted areas. Because of the large numbers of contaminants in the Great Lakes, it is virtually impossible to link carcinogenesis to a specific chemical, but circumstantial evidence for a chemical origin is strong in many cases, although it should be cautioned that viral agents and parasites can cause neoplasms.

The occurrence of liver neoplasms in brown bullhead and the levels of PAHs in sediment have been monitored for twenty years on the Black River, Ohio (Baumann and Harshbarger, 1998). In the early 1980s, the prevalence of liver cancer was high (22% to 39% in fish over 3 years old). The coke factory was closed in 1983, and by 1987 levels of PAHs in sediment had decreased by two orders of magnitude and cancer rates had fallen to one-quarter of the previous figure. Dredging of the most contaminated sediments was carried out in 1990. A subsequent marked rise in cancer rates was followed by a decrease over the next few years.

Overall, the studies involving DNA have reached an interesting stage. A great deal of medical research indicates that this information can be used to assess the impacts pollutants, especially PAHs, on wildlife.

10.4.4 Porphyrins and Heme Synthesis

Porphyrins are produced by the heme biosynthetic pathway—a vital system for most of the animal kingdom. Two major disruptions of heme biosynthesis by environmentally important agents have been studied. These are the formation of excess porphyrins after exposure to some organochlorines (OCs) and the inhibition by lead of the aminolaevulinic acid dehydratase (ALAD) enzyme.

Heme biosynthesis is normally closely regulated, and levels of porphyrins are ordinarily very low. Hepatic porphyria is characterized by massive liver accumulation and urinary excretion of uroporphyrin and heptacarboxylic acid porphyrin. Although the mechanism of OC-induced porphyria has not been completely elucidated, several researchers consider inhibition of the uroporphyrinogen decarboxylase enzyme the proximal cause. The two OCs most involved in inducing porphyria are hexachlorobenzene (HCB) and the PCBs. Although HCB has been shown in both mammals and birds to induce porphyria, the dosages required are high compared with environmental levels. PCBs have also been shown to be potent inducers, although their various congeners act quite differently.

Studies of the Rhine River showed that the patterns of hepatic porphyrins were markedly different and that the total porphyrin levels were much higher in pike collected there than in those from the cleaner River Lahn. The levels of organochlorines were up to 40-fold higher in the fish from the Rhine.

The variation in the means of the hepatic levels of highly carboxylated porphyrins (HCPs) in seven species (five orders) of birds was only twofold, and the total range was 4 to 22 pmol/g (Fox et al., 1988). No similar study appears to have been carried out on other classes of organisms. Baseline data collected from areas of low contamination show only small variations, but in view of the variability of responses to OCs in experimental studies, variability in areas of high contamination is a problem. Nevertheless, the levels of hepatic HCPs were markedly elevated in herring gulls (*Larus argentatus*) collected from the North American Great Lakes when compared with those from the Atlantic coast (Fox et al., 1988).

Aminolaevulinic acid dehydratase (ALAD) is an enzyme in the heme biosynthetic pathway. Inhibition of ALAD was first studied over thirty years ago as a means of detecting environmental lead exposure in humans and has since become the standard bioassay for this purpose. It has also been used in wildlife investigations. The assay is highly specific for lead because other metals are 10,000 times less active in causing inhibition. ALAD inhibition is rapid, but the effect is only slowly reversed. ALAD values return to normal only after about four months.

Inhibition of ALAD has been used as an indicator of lead exposure for in general situations such as urban areas and along highways, and also specifically to study lead shot in waterfowl. A threefold difference in blood ALAD activity was found between rats in a rural area and rats in an urban site in Michigan (Mouw et al., 1975). The main physiological indications of lead toxicity in the urban rats were increases in kidney weight and the incidence of intranuclear inclusions. Both effects could be correlated with lead levels. Similarly, marked differences in ALAD activity were found among feral pigeons (*Columbia livia*) from rural, outer urban, suburban, and central London areas (Hutton, 1980).

The lead levels, ALAD activity, and reproductive success of barn swallows (*Hirundo rustica*) and starlings (*Sturnus vulgaris*) along North American highways with different traffic densities were studied (Grue et al., 1986). They found a significant increase of the lead levels in the feathers and carcasses of both adults and nestlings and a 30% to 40% decrease in plasma ALAD activity. However, the number of eggs laid, the number of young fledged,

and prefledgling body weights were not affected, indicating that lead from automotive emissions does not pose a serious hazard to birds nesting near motorways.

Mortality of ducks and other waterfowl caused by the ingestion of lead shot has been a serious concern for many years. The issue was first raised in North America over seventy years ago. Ducks and geese ingest spent lead shot during the course of feeding. A nationwide survey found that 12% of the gizzard samples examined contained at least one lead shot and noted that 2% to 3% of all waterfowl in North America died from lead poisoning. Secondary poisoning of bald eagles feeding on waterfowl is another concern. National surveys of eagles found dead in the US showed that about 5% died from lead poisoning. The inhibition of ALAD has been shown to be sensitive enough to detect the effect of a single pellet. Many researchers found a strong negative correlation between blood lead concentration and log ALAD activity. The ALAD assay is simple and involves no expensive equipment or lengthy training. ALAD inhibition represents one end of the biomarker spectrum. It is a sensitive, dose-dependent measurement that is specific for a single environmental pollutant: lead.

10.4.5 Induction of Vitellogenin

The discovery of hermaphrodite roaches in stretches of rivers near sewage outlets triggered research on the effects of estrogenic disruptors in fish because natural hermaphroditism is assumed to be minimal. Examination of these fish showed that males contained high levels of vitellogenin—an egg yolk protein usually produced only by females. Thus increased levels of vitellogenin in male fish provided the basis for a biomarker assay for studies of endocrine disruptors (Box 10.1).

10.4.6 Behavioral Biomarkers

The behavioral effects of pollutants were discussed in Section 8.4.2. Use of behavioral effects in the development of biomarker assays represents a higher organizational level than others considered to date. One of the early proponents of the value of behavioral toxicology stated that the behavior of an organism represents the final integrated result of a diversity of biochemical and physiological processes. Thus a single behavioral parameter is generally more comprehensive than a physiological or biochemical parameter. Although much interesting work has been carried out in recent years, behavioral biomarkers are still not accepted as components of formal testing procedures.

Two fundamental difficulties surround the use of behavioral tests in wildlife toxicology. First, the best studied and most easily performed and quantified tests exhibit the least environmental relevance. Second, the most relevant behaviors are the most strongly conserved against change (Peakall, 1985).

Operant behavior such as conditioning to respond to a colored key to obtain food is too remote from real life to relate to survival. It can merely be presumed that a decrease in learning ability is an unfavorable response. Avoidance behavior is more directly related to survival, although the relationship has not been quantified. The ability to capture food is clearly important to predatory species but is difficult to measure under field conditions.

Field observations are difficult to quantify. It was suggested that behavioral changes possibly led to the decline of the peregrine falcon. However, observations by time-lapse photography at the eyries of highly contaminated peregrines revealed little abnormal behavior (Enderson et al., 1972). This study was based on seven peregrine eyries in

BOX 10.1 INDUCTION OF VITELLOGENIN IN FISH

A survey of five rivers in England was conducted in the summer of 1994 (Harries et al., 1997). Caged male rainbow trout were deployed at five sites in each river, one upstream from the suspected sources (waste treatment plants), one at the point of effluent discharge, and the other three at different distances downstream.

In four cases, the fish placed in the neat effluent showed very marked and rapid increases in vitellogenin levels. In two cases, none of the downstream sites showed estrogenic activity; in another, activity was detected 1.5 km downstream. The fourth river was quite different: the effluent was extremely estrogenic and so was water sampled at all other sites (maximum distance 5 km). The effluents contained much higher levels of alkylphenolic compounds. On the fifth river, even the neat effluent did not cause increased levels of vitellogenin. In this case, the small waste treatment plant did not receive industrial waste.

A disturbing finding is that some UK estuaries show high degrees of estrogenic contamination. Flounder (*Platichthys flesus*) in the Tyne and Mersey estuaries had vitellogenin levels four to six orders of magnitude higher than controls (Scott et al., 1999). Elevation of vitellogenin was less marked in the Crouch and the Thames.

The implications of these findings at the population level are not known and are difficult to determine. Sewage discharges may well contain other compounds that cause reproductive toxicity by other means. The effect on lowland coarse fish may well be different from effects on trout, which usually do not occur in these waters. It would be important to examine estuaries for effects on fish that reproduce in these areas rather than the flounder that breeds at sea. Myriad other factors also affect wild populations of fish. For further discussion, see Section 15.2. and Chapter 15 in Walker (2009).

Alaska, using battery-powered time-lapse motion picture cameras that took pictures about every 3 min. The film cartridges were replaced every 6 or 7 days. Replacement was difficult because the eyries were widely separated and the terrain was difficult. In two of the nests, the eggs broke, but no evidence of abnormal behavior was observed. The other five nests were successful. In all, some 70,000 pictures covering 4,200 hours were obtained. One of the drawbacks of this type of experiment is the time required to analyze the data obtained.

In another study (Nelson, 1976), 300 hours of observations of twelve peregrine eyries from a hide were analyzed. Four clutches lost single eggs, probably by breakage, but no abnormal behavior was observed. Although 300 hours is a lot of observation time, the average observation per clutch was only 25 hours among 400 total hours of daylight during the incubation period. These two studies illustrate the difficulties of observing behavioral changes in the field. Even if behavioral changes are documented, it is difficult to relate them to specific chemicals.

The best documented studies that can be extended to real life situations are those involving the organophosphorous pesticides and the subsequent inhibition of AChE. These were covered in Section 8.4, and their use in field conditions is discussed further in Section 16.4.

Studies of avoidance responses of fish to toxicants dates back over eighty years. From the first simple studies on acetic acid, the field has grown enormously, and the equipment has

become highly complex, for example, sophisticated fish avoidance chambers with video monitors and computer-interfaced recording systems.

Recent studies include many on the effects of heavy metals. A comparison of the lowest observed effect concentration (LOEC) based on behavioral studies (avoidance, attraction, fish ventilation, and cough rates) with chronic toxicity studies indicates that some of the behavior tests are more sensitive than life cycle or early life stage tests. Other studies involving predator avoidance, feeding behavior, learning, social interactions, and locomotor behaviors have been insufficient to allow judgments about their sensitivity or utility.

At present, behavioral tests have not replaced conventional toxicity tests. However, they may provide ecological realism, e.g., the effects of pollutants on predator–prey relationships but they must be capable of field validation.

The behavioral tests that are the most advanced involve fish. The fish avoidance test is well established in the laboratory as a means of showing effects well below the lethal range, and highly automated procedures are available. Nevertheless, a note of caution should be injected. Pre-exposure to effluent reduced the avoidance behavior, and pre-exposed fish were observed more often in contaminated than in clean water (Hartwell et al., 1987). This desensitization caused by pre-exposure makes it likely that laboratory experiments will overestimate the responsiveness of fish to metal pollution in the wild.

These difficulties do not imply that behavioral effects caused by pollution are unimportant. Studies such as those examining the predation pressure on fiddler crabs (*Uca pugnax*) have shown that operational levels of pesticide use can cause population effects through behavioral changes (Ward et al., 1976). Substandard prey are more readily captured by predators, but field studies of the impact of chemicals on behavior are difficult.

The overall conclusion is that behavioral parameters are not especially sensitive to exposure to pollutants and that biochemical and physiological changes are usually at least as sensitive. Further, the variability of biochemical data is generally less and the dose–response relationship clearer than those obtained from behavioral studies. In general, physiological and biochemical changes are more readily measured and quantified.

10.4.7 Biomarkers in Plants

Plants have been widely used as biomonitors to localize emission sources and analyze the impacts of pollutants, especially gaseous air pollutants, on plant performance. One of the earliest studies was by Angus Smith, who examined the damage to plants around Manchester from what he called *acid rain*. However, biomarkers should go beyond the visible parameters of sentinel species. They should establish processes and products of plants that will allow recognition of environmental stress before damage is apparent. A biomarker must be able to predict an environmental outcome and consequential damage. Ideally, biomarkers should be selected from the events of biochemical or physiological pathways, but reaching this stage with plant biomarkers is difficult (Ernst and Peterson, 1994).

Specific biomarkers have been identified in sensitive plants. In a few cases, excess of a specific chemical will lead to the production of a metabolite that differs in tolerant and sensitive plants. In the presence of excess selenium, Se-sensitive plants fail to differentiate between S and Se. They incorporate Se in sulfur amino acids, leading to the synthesis of enzymes of lower activity that can lead to plant death. In contrast, Se-tolerant plants biosynthesize and accumulate nonprotein selenoamino acids such as selenocystathionein and Se-methyl-selenocysteine that do not cause metabolic problems. Thus selenoproteins in plants are excellent biomarkers for Se stress, although their use in the field has not been widely reported.

Another example is that after exposure to excess fluor (a mineral containing fluorine), plants synthesize fluoroacetyl coenzyme A and then convert it via the tricarboxylic acid cycle to fluorocitrate—a compound that blocks the metabolic pathway by inhibiting the aconitase enzyme. As a result, fluorocitrate accumulates and is a very reliable biomarker for fluor poisoning.

Some plant biomarkers reveal free metals. Phytochelatins are synthesized during exposure to a number of metals and anions such as SeO_4^{2-}, SeO_3^{2-}, and AsO_4^{3-}. Dose- and time-dependent relationships have been established under laboratory conditions for cadmium, copper, and zinc. For monitoring purposes, research is needed into the phytochelatin production of plants in the field.

General plant biomarkers that respond to a variety of environmental stresses may be useful to indicate that some component in the environment is a hazard to plant life. For example, the activity of the peroxidase enzyme has been used to establish the exposure of plants to air pollution, especially to SO_2.

Changes of enzyme systems during the development stage of plants and the effects of seasonal and climatic processes are not yet sufficiently known to demonstrate the reliability of enzyme activity as a monitoring device. At present, plant biomarkers are not as well advanced as animal biomarkers, but this is likely to change. The stationary nature of plants aids greatly in measuring exposure to pollutants; monitoring surveys for measuring levels of metals in lichens and organochlorines in pine needles are in place and would be more valuable if the measurements could be linked with biological changes in the plants.

10.5 Role of Biomarkers in Environmental Risk Assessment

The most compelling reason for using biomarkers in environmental risk assessment is that they yield information about the effects of pollutants. Thus their use in biomonitoring is complementary to the more usual monitoring by determining or predicting residue levels (see Section 6.5 for a discussion of risk assessment).

The first point in any assessment process is to decide what to assess. This may sound self-evident, but it is surprising how seldom precise objectives are defined. This is the case with many monitoring programs intended to determine levels of environmental chemicals. Take, for example, the International Mussel Watch Programme that measures metals in mussels in many parts of the world. The justification of such surveys is that we should know what pollutants are where and at what concentrations. However, what will be done with the information? Only if we know what concentrations are hazardous and what effective remedial action can be taken will such information be of practical use.

Similar considerations apply to biomarkers. Again, action levels must be determined if a monitoring program is to be effective. An advantage of the biomarker approach is that it may show that the physiology of an organism is within normal limits, indicating that no action is necessary. By contrast, zero levels are rarely found in analytical determinations. Ideally, both approaches (i.e., residues and biomarkers) should be utilized in an integrated manner (Chapter 11).

Before legislation to limit risks can be enforced, two fundamental questions must be answered: (i) the amount of damage we are prepared to tolerate and (ii) the amount of

proof required. At one extreme, little concern will arise if a few aquatic invertebrates die within a few meters of the end of an outlet pipe. At the other end of the scale, events such as the destruction of most of the biota of the Rhine River (Deininger, 1987) set off a worldwide reaction. Our concerns are also species dependent and tend to increase as we move from algae to mammals and widespread differences exist even within classes of animals. It is easy to arouse concern about pandas and whales and difficult in the case of rats. Further, some types of damage are considered more serious than others; alteration of the genetic material that may be passed onto future generations is considered one of the most serious effects.

The question of how much damage we are prepared to tolerate is for society to answer; then it will be possible to design protocols to meet the standards required. Without an answer, scientists face the problem of trying to set up regulations without a goal. The question of how much proof is enough is largely scientific and it cannot be answered until the question about tolerable damage is answered. Despite the inertia, decisions must be made. Failure to make a decision constitutes making a decision.

Why use biomarkers in risk assessment? One important reason arises from the limitations of classical risk assessment. The basic approach is to measure the chemical present and then use animal experiment data to relate the chemical data to the adverse effects caused (Section 6.5). The limitation of this approach is that we have defined the levels critical to organisms for very few compounds. Under real life conditions, many organisms are exposed to complex and changing levels of mixtures of pollutants. Chemical monitoring works only if a material is persistent. Chemicals such as the PAHs and many pesticides have very short biological half-lives in most species but may nevertheless exert long-term effects. Biological and chemical monitoring systems should be complementary; we must know what chemicals are where and what their effects are.

The first question that biomarkers can help answer is whether environmental pollutants are present at a sufficiently high concentrations to cause effects. If the answer is positive, further investigation to assess the nature and degree of damage and the causal agents is justified. A negative answer means that no further resources must be invested. In this way, biomarkers can act as important early warning systems.

The role of biomarkers in environmental risk assessment is determining whether organisms in a specific environment are physiologically normal. The approach resembles the study of biochemistry in humans. A suite of tests can be carried out to see whether an individual is healthy. It is necessary to select both the tests and the species to be tested.

In selecting tests, the specificity of a test to pollutants and the degree to which the change can be related to harm must be considered. Both specific and nonspecific biomarkers are valuable in environmental assessments. In an ideal world, we would have biomarkers to indicate exposures to and assess risks of all major classes of pollutants and nonspecific biomarkers to assess accurately and completely the health of organisms and their ecosystems.

Clearly, the definition of harm must cover scientific and social aspects. Scientifically, it is important to demonstrate unequivocally that changes resulted from pollution, and biomarkers have an important role to play. Whether a change is sufficiently serious to warrant the cost of remedial action is for society to decide. These issues will be taken further in Chapter 17 of this book.

10.6 Summary

A biomarker is defined as a biological response to an environmental chemical at the individual level or below that reveals a departure from normal status. The specificity of biomarkers to pollutants varies greatly. Highly specific biomarkers are valuable for detecting exposure to and possible effects of specific chemicals but yield no information about other pollutants. In contrast, nonspecific biomarkers show that exposure to pollutants has occurred without identifying the pollutant responsible. A number of specific biomarkers were considered in some detail. Biomarker assays are particularly useful when they relate to toxic effect (mechanistic biomarkers)—not just to exposure. The most important reason for using biomarkers in environmental risk assessment is that they demonstrate the effects of pollutants; their use is complementary to biomonitoring that involves the determination of levels of environmental chemicals.

Further Reading

Fossi, M.C. and Leonzio, C., Eds. (1994). *Nondestructive Biomarkers in Vertebrates*. Proceedings of a workshop devoted to the use of blood and other tissues that can be collected nondestructively to measure biomarkers.

Huggett, R.J. et al. (1992). *Biomarkers. Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*. Techniques and extensive coverage of DNA alterations and immunological biomarkers.

McCarthy, J.F. and Shugart, L.R. (1990). Compilation of papers presented by American and European scientists at an American Chemical Society meeting.

Peakall, D.B. (1992). *Animal Biomarkers as Pollution Indicators*. Use of biomarkers of higher animals for environmental assessment.

Peakall, D.B. and Shugart, L.R. eds. (1993). *Biomarkers: Research and Application in the Assessment of Environmental Health*. NATO symposium.

Peakall, D.B. et al. (1999). *Biomarkers: A Pragmatic Basis for Remediation of Severe Pollution in Eastern Europe*. NATO workshop.