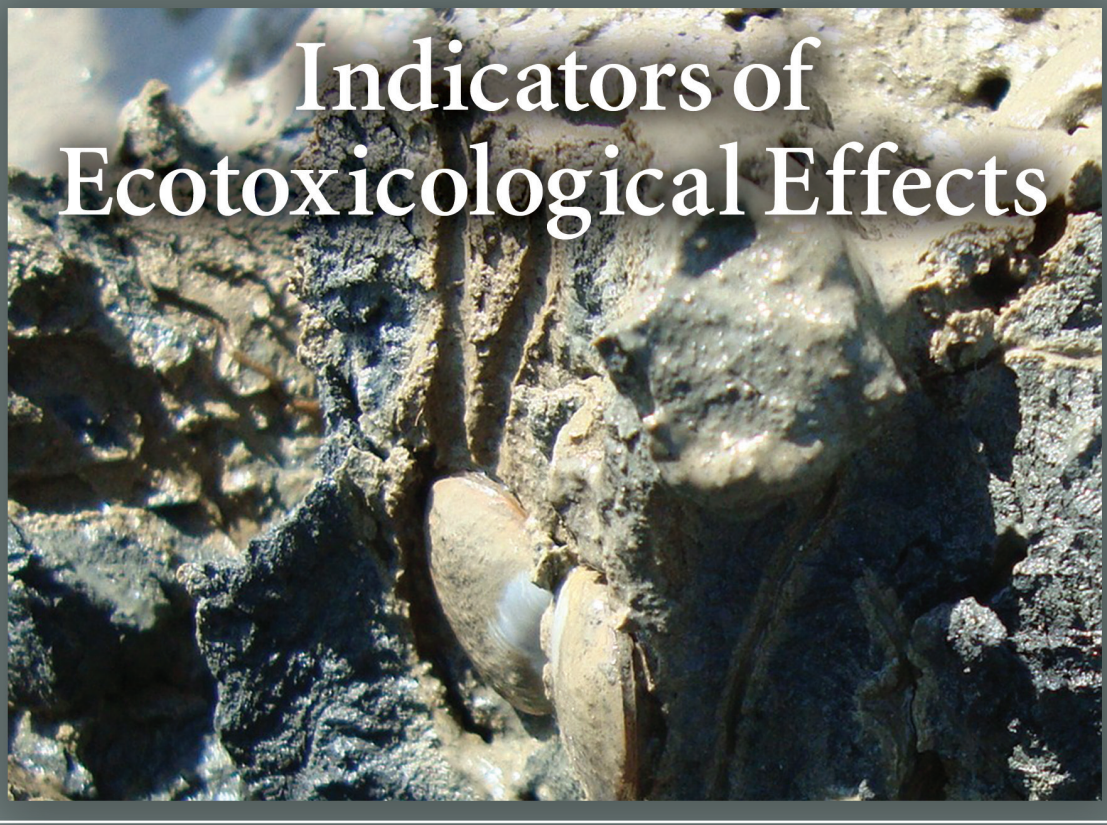
Ecological Biomarkers

Indicators of Ecotoxicological Effects



Edited by
Claude Amiard-Triquet
Jean-Claude Amiard
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CRC Press
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Preface

Aims and Scope

The biomarker concept was initially developed with the medical purpose of the early diagnosis of pathological status and for use in mammalian toxicology. At the beginning of the 1990s, ecotoxicologists became interested in the concept, which stimulated important debate, for instance, at the 2nd European Conference on Ecotoxicology organized by the Society of Ecotoxicology and Environmental Safety (SECOTOX) in Amsterdam in 1992. In 1994, Depledge proposed a definition that is still authoritative today: "A biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)."

In the United States, the Clean Water Act is the primary federal law governing water pollution. Because of its statutory responsibilities, the US Environmental Protection Agency has developed a strategy to improve monitoring and assessment of environmental risk in aquatic ecosystems at local, state, regional, and national scales. In this framework, the Environmental Monitoring and Assessment Program (EMAP) has substantially advanced the scientific basis for monitoring the condition of aquatic ecosystems. The EMAP strategy includes physicochemical indicators in sediments and the water column and, for biological indicators, mainly responses at the level of the community. The Water Framework Directive (WFD) promulgated by the European Parliament and Council is the chosen way forward to maintain or improve the quality of European aquatic environments. In this aim, it is necessary to attain a good status of these waters. This good status is based on both the chemical and the ecological status of the water masses. The chemical status is considered "good" when the concentrations of chemicals in the medium are below the limits defined in EC's regulations. The characterization of the ecological status of water masses is mainly based on the composition and abundance of certain plant and animal taxa. The failure of the WFD to recognize a role for biomarkers in this context is regrettable as is their limited use in the EMAP strategy.* By neglecting biomarkers, both regulatory bodies ignore a category of biological tools well known to be precocious and sensitive indicators of the degradation of organism health. Effects at the community level allow an ecotoxicological assessment after severe environmental degradation has already occurred, thus leading to expensive remediation processes, whereas biomarkers have an interesting potential as predictive tools usable much earlier in any environmental degradation process.

Ecological analyses recommended in the EMAP or the WFD are useful to describe differences between sites, differently impacted by anthropogenic pressure, or to reveal temporal changes when historical records are available. However, ecological approaches are of no help in determining the origin of such changes, whereas so-called "specific" biomarkers can contribute to answering this type of question. Some biomarkers are currently used for the implementation of the OSPAR Convention for the Protection of the Marine Environment of the

* USEPA, July 2002. EMAP research strategy. Report EPA 620/R-02/002.

Northeast Atlantic, such as those for metal-specific biological effect monitoring (e.g., metallothionein, δ-amino levulinic acid dehydratase inhibition in blood [ALA-D]) and PAH-specific biological effect monitoring (e.g., cytochrome P4501A, DNA adducts).

Chemical data needed to fulfill the requirements of the WFD or the EMAP strategy may be useful to predict the potential effects on living organisms but only if the dose–effect relationship is well established. Predicted No Effect Concentrations can be derived from laboratory toxicological tests, but the main limit of this practice is that toxicity data are nearly always determined for individual chemicals, whereas in real life numerous molecules or classes of molecules coexist in waters with the possibility of multiple interactions. Among these toxic compounds (including numerous persistent organic pollutants), many are not yet analytically accessible or are analyzable only at exorbitant cost. Thus, it is necessary to develop other strategies to assess the degree to which a given ecosystem is impacted or not by toxic contaminants. In attempting to fulfill this aim, "generalist" biomarkers can reveal the integrated ecotoxicity of complex mixtures, particularly physiological markers linked to the growth and reproduction of organisms.

At the end of the 1990s, several books established the state of the art of biomarker methodology, such as *Use of Biomarkers for Environmental Quality Assessment*, published by Science Publishers, Enfield, USA, in 2000 (Lagadic, Caquet, Amiard and Ramade, eds.). However, as mentioned above, the use of biomarkers remains comparatively marginal in ecological risk assessment. Several reasons may be responsible for this. In the first issue of the journal *Ecotoxicology* (1992), Cairns pointed to one of them, termed the "signal-to-noise ratio." If the natural variation of a given biomarker is weak in the absence of chemical stress, the change induced by chemical stress will be easily detectable. On the other hand, significant natural variation in a biomarker has the potential to conceal—at least partly—a stress-induced additional variation. However, the question of such confounding factors (season, age, sex, etc.) is not peculiar to the methodology of biomarkers and has been mastered (using adapted sample strategies and statistical treatments) in the framework of Mussel Watch programs, based on the monitoring of pollutant concentrations in biological matrices. A second reason for the lack of wider take-up of the use of biomarkers appeared when it became clear that several biomarkers previously considered specific (e.g., decrease of AChE activity in the presence of organophosphate pesticides and carbamates) were also found to be responsive to other molecules (metals, algal toxins) or other forms of stress. Lastly, both specific and generalist biomarkers are determined at the individual or suborganismal level. Does a change that affects a few biological macromolecules, some cells, or a few individuals within a population have any ecological significance that would allow the prediction of deleterious effects at higher levels of biological organization, namely, the population, community, and ultimately the ecosystem?

Over the past decade, the importance of developing biomarkers with added ecological value has been recognized. Subsequent to the publication of our first book, *Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques*, published by Lavoisier, Paris, in 2008 (Amiard and Amiard-Triquet, eds.), it is time to revisit those biological responses that are the most ecologically relevant in order to diagnose degradation of the health status of an aquatic environment well before it becomes unmanageable. The literature reviewed in this book supports the efficacy of the use of lysosomal biomarkers, immunotoxicity effects, behavioral disturbances, energy metabolism impairments, endocrine disruption measures, and genotoxicity as all indicative of probable toxic effects at higher biological levels. These biomarkers thus provide a real possibility of delivering the holy grail—an easily measured biomarker at a simple level of biological organization that is predictably linked to a potentially ecologically significant effect at higher levels of biological organization. This book provides the burning torch to light our way in this quest.

Editors

Claude Amiard-Triquet is a research director in the CNRS (French National Research Center) based at the University of Nantes, France. She earned the degree of DSc in 1975 for her research in radioecology at the French Atomic Energy Commission. Dr. Amiard-Triquet's topics of research interest include metal ecotoxicology, biomarkers, and, more recently, emerging contaminants (endocrine disruptors, nanoparticles). As the head of multidisciplinary research programs, she has managed research collaborations between specialists in organic and inorganic contaminants and chemists and biologists involved in studies from the molecular to ecosystem levels, with a constant concern for complementarity between fundamental and applied research. Dr. Amiard-Triquet regularly acts as an expert for the assessment of scientific proposals (e.g., the European Framework Program for Research and Development, the International Foundation for Science, and the Sea Grant Administration, Oregon State) and is also in demand as a referee for a dozen or so international journals. She has authored or co-authored more than 180 research papers and has authored 27 chapters in various books. Dr. Amiard-Triquet has also co-authored one book, *La Radioécologie des Milieux Aquatiques*, with J.C. Amiard and co-edited three books: *L'Évaluation du Risque Écologique à l'Aide de Biomarqueurs* with J.C. Amiard, *Environmental Assessment of Estuarine Ecosystems: A Case Study* with P.S. Rainbow, and *Tolerance to Environmental Contaminants* with P.S. Rainbow and M. Roméo. She has given or contributed to about 100 presentations at international meetings.

Jean-Claude Amiard is a research director in the CNRS based at the University of Nantes, France. He was an associate professor at the University of Quebec at Rimouski from 1994 to 2010. He earned his DSc degree in 1978 from the University Pierre and Marie Curie, Paris. He has directed 16 PhD theses and contributes to master's teaching in several French and foreign universities. In 2011, he has gathered all this teaching material into a book, *Risques chimiques environnementaux. Méthodes d'évaluation et impacts sur les êtres vivants*. He acts as an expert for governmental organizations in charge of health security Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) or information on nuclear activities Association nationale des comités et commissions locales d'information (ANCCLI), and in this framework, he has co-edited a book, *Le tritium, actualité d'aujourd'hui et de demain*, with S. Gazal. Previously, he has co-authored and co-edited two books on biomarkers with L. Lagadic, T. Caquet, and F. Ramade and one book, *L'Évaluation du Risque Écologique à l'Aide de Biomarqueurs*, with C. Amiard-Triquet. His research activities have focused on the fate and effects of trace metals in marine and estuarine ecosystems, on the tolerance of organisms to chronic exposure to contaminants, and, more recently, on the application of biomarkers to the assessment of ecotoxicity of emerging contaminants. He has published more than 130 papers in peer-reviewed journals, 90 papers in national journals or congress proceedings, and 32 book chapters or books. He has participated in 140 national and international congresses.

Philip Rainbow is the head of the Department of Zoology at the Natural History Museum, London, leading a staff of more than 100 working scientists. He earned a PhD (1975) and a DSc (1994) from the University of Wales. Dr. Rainbow was appointed (1994) to a personal chair in the University of London, where he was head of the School of Biological

Sciences at Queen Mary (1995–1997) and is now a visiting professor. He has taught Metals in the Marine Environment at Queen Mary for more than a decade. Professor Rainbow has served as a member of the Natural Environment Research Council (NERC) Marine Science Peer Review Committee, NERC Peer Review College, the Council of the Linnean Society of London, and the Advisory Committee of the Darwin Initiative (DEFRA, UK Government). He has been an editor of the *Journal of Zoology* and is on the editorial boards of *Environmental Pollution*, *Marine Environmental Research*, and the *Journal of the Marine Biological Association UK*. In 2002, Dr. Rainbow was invited to give the Kenneth Mellanby Review Lecture by the journal *Environmental Pollution* at the Society of Environmental Toxicology and Chemistry annual meeting at Salt Lake City, Utah. He has more than 210 peer-reviewed publications, six co-edited books, and two co-authored books. The first (*Biomonitoring of Trace Aquatic Contaminants* with D.J.H. Phillips*)* went to two editions. The second, co-authored with Professor Sam Luoma, *Metal Contamination in Aquatic Environments: Science and Lateral Management*, was published in 2008 by Cambridge University Press and has now been issued in paperback. Dr. Rainbow's recent research has focused on the factors affecting the bioavailability of trace metals to aquatic invertebrates from both solution and the diet and the biodynamic modeling of trace metal bioaccumulation.

Contributors

Jean-Claude Amiard
CNRS, Université de Nantes
Mer, Molécule, Santé, EA 2160
Nantes, France

Claude Amiard-Triquet
CNRS, Université de Nantes
Mer, Molécule, Santé, EA 2160
Nantes, France

Franck André Atienzar
Responsable unité de toxicologie *in vitro*
UCB SA
Braine-l'Alleud, Belgium

Doris W. T. Au
Department of Biology and Chemistry
City University of Hong Kong
Kowloon, Hong Kong

Michel Auffret
Institut Universitaire Européen de la Mer
LEMAR UMR CNRS 6539
Plouzané, France

Brigitte Berthet
ICES and Université de Nantes
Mer, Molécule, Santé, EA 2160
Nantes, France

Pauline Brousseau
INRS–Institut Armand-Frappier
Laval, Quebec, Canada

Arnaud Chaumot
IRSTEA - UR "Milieux aquatiques, écologie et pollutions"
Laboratoire D'écotoxicologie
Lyon, France

Michael W. L. Chiang
Department of Biology and Chemistry
City University of Hong Kong
Kowloon, Hong Kong

Tracy K. Collier
Oceans and Human Health, NOAA
Bainbridge Island, Washington

Carole Cossu-Leguille
Université Paul Verlaine de Metz
CNRS UMR 7146 Laboratoire des Interactions Ecotoxicologie, Biodiversité, Ecosystèmes (LIEBE)
Metz, France

Mickaël Couderc
Université de Nantes
Mer, Molécule, Santé, EA 2160
Nantes, France

Odile Dedourge-Geffard
Université Reims Champagne Ardenne
Unité Interactions Animal-Environnement EA4689
UFR Sciences Exactes et Naturelles
Reims, France

Françoise Denis
Université du Maine - Muséum National d'Histoire Naturelle
Département Milieux et Peuplements Aquatiques, UMR 5178 "BOME"
Concarneau, France

Cyril Durou
CEHTRA
Sainte Eulalie, France

Michel Fournier
INRS–Institut Armand-Frappier
Laval, Québec, Canada

Héloïse Frouin
Institute of Ocean Sciences (Fisheries and Oceans Canada)
Sidney, British Columbia, Canada

François Gagné
Section Recherche sur les Écosystèmes Fluviaux
Direction de la Recherche pour la Protection des Écosystèmes Aquatiques
Science et Technologie de l'Eau, Environnement Canada
McGill, Montréal, Québec, Canada

Jeanne Garric
IRSTEA, Laboratory of Ecotoxicology and Biology
Lyon, France

Alain Geffard
Université de Reims Champagne Ardenne
Unité Interactions Animal-Environnement EA 4689
UFR Sciences Exactes et Naturelles
Reims, France

Olivier Geffard
IRSTEA, Laboratory of Ecotoxicology and Biology
Lyon, France

Laure Giambérini
Université Paul Verlaine de Metz
CNRS UMR 7146 Laboratoire des Interactions Ecotoxicologie, Biodiversité, Ecosystèmes (LIEBE)
Metz, France

Matthew J. Gubbins
Marine Scotland Science, Marine Laboratory
Aberdeen, Scotland

Martial Huet
Université de Bretagne Occidentale
Institut Universitaire Européen de la Mer
LEMAR UMR CNRS 6539
Plouzané, France

Jean Laroche
Université de Bretagne Occidentale
Institut Universitaire Européen de la Mer Laboratoire des Sciences de l'Environnement Marin
LEMAR UMR CNRS 6539
Plouzané, France

Sébastien Lemière
Maître de conférences des universités
Université des Sciences et Technologies de Lille
Laboratoire "Ecologie numérique et Ecotoxicologie"
Villeneuve d'Ascq, France

Reinier M. Mann
Hydrobiology, Consulting Company
Auchenflower, Queensland, Australia

Justine Marchand
Université du Maine (Le Mans)
Mer, Molécule, Santé, EA 2160
Le Mans, France

Christophe Minier
Laboratory of Ecotoxicology
University of Le Havre
Le Havre, France

Michael N. Moore
European Centre for Environment and Health
Peninsula College of Medicine and Dentistry
Universities of Exeter and Plymouth
Truro, England

Catherine Mouneyrac
CEREA, Université Catholique de l'Ouest
Université de Nantes, Mer, Molécule, Santé, EA 2160
Nantes, France

Frédéric Palais
Université Reims Champagne Ardenne
Unité Interactions Animal-Environnement EA4689
UFR Sciences Exactes et Naturelles
Reims, France

Alexandre Péry
INERIS, Unité Modèles pour l'Écotoxicologie et la Toxicologie
Verneuil-en-Halatte, France

Stéphane Pillet
Research Institute of the McGill University Health Center
Montreal, Quebec, Canada

Philip S. Rainbow
Department of Zoology
The Natural History Museum
London, England

François Rodius
Maître de Conférences des universités
Université Paul Verlaine Metz
CNRS UMR 7146
Metz, France

Michèle Roméo
Chargée de Recherche INSERM, Université de Nice Sophia-Antipolis
Faculté des Sciences, EA ECOMERS
Nice, France

Susanna Sforzini
Department of Science and Technological Innovation (DiSIT)
University of Piemonte Orientale "A. Avogadro"
Alessandria, Italy

Paul Somerfield
Plymouth Marine Laboratory
Plymouth, England

Paule Vasseur
Université Paul Verlaine de Metz
CNRS UMR 7146 Laboratoire des Interactions Ecotoxicologie, Biodiversité, Ecosystèmes (LIEBE)
Metz, France

Aldo G. Viarengo
Department of Science and Technological Innovation (DiSIT)
University of Piemonte Orientale "A. Avogadro"
Alessandria, Italy

Benoît Xuereb
Laboratory of Ecotoxicology
University of Le Havre
Le Havre, France

1

Introduction

Claude Amiard-Triquet and Jean-Claude Amiard

CONTENTS

Anthropogenic activities are responsible for the environmental input of many classes of chemicals through industrial sources, domestic and urban effluents, and diffuse sources linked to agriculture. The main categories of contaminants include both organic [petroleum hydrocarbons, polychlorobiphenyls (PCBs), pesticides, etc.] and inorganic (metals and nonmetallic elements) compounds. These compounds were studied as soon as ecotoxicology appeared as a specific branch of environmental studies, whereas emerging contaminants have become a topic of concern more recently, even though some of them have been present in the environment for years. Emerging contaminants include pharmaceutical and care products, alkylphenols, brominated flame retardants, perfluorinated organic compounds, and nanoparticles.

Depending on their physical characteristics, three main categories may be distinguished among chemical wastes: solids, liquids, and gases. Each category corresponds to one of the compartments of our physical environment: lithosphere, hydrosphere, atmosphere. However, it is impossible to describe chemicals entering our environment as continental, aquatic, or atmospheric contaminants since many exchanges occur between these compartments. Whatever the point of entrance of a given substance into the environment, an important fraction may be carried over what may be a significant distance as a result of water and air circulation. As a consequence, even polar environments are not spared, and in a charismatic species such as the polar bear, increasing levels of persistent organic pollutants are well documented, with possible ecotoxicological effects at the population level (Letcher et al. 2010).

Even if contaminants are distributed on a worldwide scale, dilution in air or water masses increases with distance from the contamination source. This contamination gradient is the primary factor controlling contaminant uptake into organisms (Figure 1.1). Environmental conditions influence the transformation of many chemicals through chelation, hydrolysis, photodegradation, biodegradation, etc. However, some degradation products of contaminants are not less toxic than the initial molecule, sometimes being even more noxious.

Many toxicants are able to cross biological membranes but these membranes and associated structures can act as barriers to contaminant entry (Figure 1.1). For instance, metal speciation and therefore dissolved metal bioavailability may be modified through ligand secretion into the external medium or by precipitation of dissolved metals as microcrystals of metal sulfides onto the cell surface. Secretion of exudates by a variety of organisms (bacteria, plants, animals) can involve a great variety of compounds. Subtle changes in the charge and types of reactive groups in such secretions can interfere markedly with

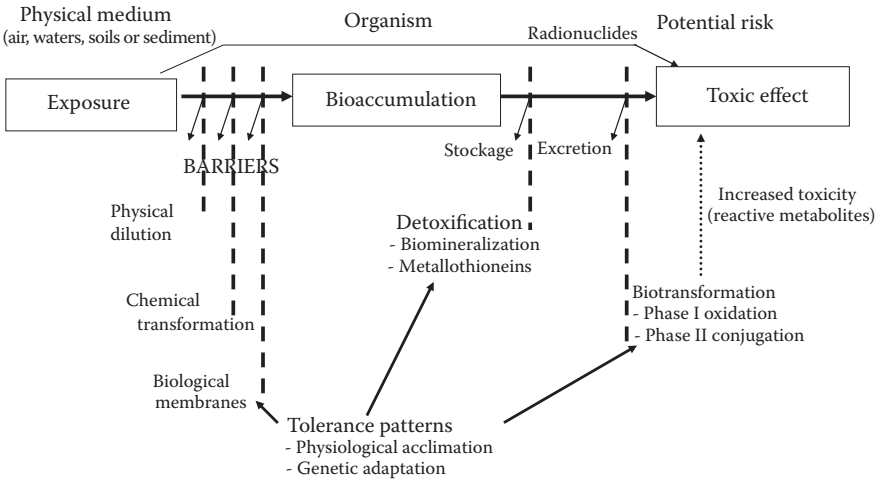

FIGURE 1.1
The ecotoxicology triad.

their metal binding characteristics and consequently the biological uptake of the metal. Another mechanism of limiting contaminant uptake is the existence of impervious extracellular barriers such as cuticles, integuments, tests, shells, and scales that contribute to reduce the cell epithelial surface available to contribute to transepithelial transport (for details, see Mason and Jenkins 1995).

Once incorporated into an organism (Figure 1.1), contaminants can be either stored in tissues or excreted. Storage in intra- or extracellular compartments does not necessarily result in a toxic effect in organisms. For instance, metal detoxification is efficient in numerous organisms. It may be based on the synthesis of metallothioneins (MTs), a family of metalloproteins able to sequester metals via metal binding to their constituent thiol groups, thus blocking any interference between the metals and enzymes that would otherwise result in subsequent enzymatic activity impairments. MT induction is the most common toxic metal defense mechanism in vertebrates. It is also present in most biological taxa (Amiard et al. 2006), but among invertebrates, the major mode of metal detoxification is metal biomineralization in various types of cellular inclusions (Marigomez et al. 2002). It is only when the metal-binding capacity of these ligands is overwhelmed that metal toxicity can occur.

On the contrary, processes responsible for excretion are not systematically free of noxious effects on organisms. Biotransformation of certain organic pollutants [polycyclic aromatic hydrocarbons (PAHs), PCBs] is organized into two phases (Figure 1.1). Phase I reactions consist of oxidation, reduction, and hydrolysis processes. Phase II enzymes serve to link metabolites from phase I with endogenous substrates, increasing their water solubility and thereby facilitating their excretion. However, phase II biotransformation sometimes leads to reactive metabolites, the interactions of which with cellular macromolecules can engender toxicity (Roméo and Wirgin in Amiard-Triquet et al. 2011). Biotransformation is followed by phase III leading to the elimination of metabolites by the multixenobiotic transport system (Damiens and Minier in Amiard-Triquet et al. 2011).

The activity of biotransformation enzymes (such as cytochrome P450 enzymes, including ethoxyresorufin *O*-deethylase involved in phase I; glutathione *S*-transferase involved in phase II) or MT concentrations are some examples of biomarkers that have been proposed

to assess the exposure of organisms to contaminants present in their environment (Chapter 2). In addition to inducing MT synthesis or activating cytochrome P450 enzymes, metals, PCBs, and PAHs can increase oxidative stress by increasing the concentrations of reactive oxygen species naturally present in organisms. Cytotoxicity can occur, including lipid peroxidation and DNA damage, but the degree of such damage depends on the efficiency of enzymatic (superoxide dismutase, catalase, glutathione peroxidase, etc.) and nonenzymatic defenses. If DNA damage induced by metabolites resulting from contaminant biotransformation is not adequately repaired by specialized nuclear enzymes, this can lead to an erroneous expression of the genome, including the activation of oncogenes, which constitutes the first step of the transformation of a normal cell in a tumoral cell (Newman and Clements 2008).

As an indicator of neurotoxicity effects, acetylcholinesterase (AChE) activity has been initially considered a specific biomarker of exposure to organophosphate and carbamate pesticides. More recently, however, other groups of chemicals present in the marine environment including metals, detergents, hydrocarbons, and also cyanobacterium toxins have been shown to affect AChE activity (Table 4.1).

This lack of biomarker specificity poses a problem for environmental management. Although biomarkers are able to reveal the presence of contaminants, and subsequent changes in the biology of organisms, any lack of specificity in their response reduces the likelihood of precise targeting of a particular contaminant, thereby affecting management decisions to reduce contamination and its impacts. To date, only a few biomarkers seem really specific: δ-amino levulinic acid dehydratase inhibition in blood able to reveal lead contamination, bile fluorescent compounds for petroleum hydrocarbons (Anderson and Lee 2006), and imposex in gastropod mollusks in response to TBT contamination (Chapter 9). However, less specific biomarkers are also interesting environmental management tools as general responses to the degradation of environmental conditions, and they are still important in assessing the health status of a given medium exposed to chronic or acute (e.g., oil spill) pollution pressure. Among these biomarkers, stress proteins, which contribute to cellular protection and are highly conserved throughout evolution from bacteria to humans, can provide information on a large spectrum of environmental stress (Newman and Clements 2008). Histological alterations generally result from the integration of biochemical and physiological changes that may be caused by various chemical contaminants (Newman and Clements 2008). Until now, no immune response specific for a given contaminant has been described, but this category of biomarkers is useful in detecting effects linked to simultaneous exposure to multiple contaminants (Fournier et al. 2005). Lastly, a variety of nonspecific biomarkers are important because they are involved in growth and development and contribute to the success of reproduction with possible ecological consequences on population sustainability and ecosystem functioning when key species are impacted. To aggregate the benefit of specific, less specific, and general biomarkers, it is generally recommended to date to use biomarkers in a battery for ecological risk assessment, as recommended, for instance, by Anderson and Lee (2006) and Thain et al. (2008) in oil spill risk assessment (Chapter 2).

Classically, biomarkers have been classified as biomarkers of exposure, effect, and susceptibility (Manahan 2003). However, the definitions of these classes vary depending on different authors (Chapter 2). So, certain ecotoxicologists prefer the terminology proposed by De Lafontaine et al. (2000), contrasting biomarkers of defense (Chapter 3) and biomarkers of damage (Chapters 4–6).

Biomarkers of defense include MTs, phase I, II, and III enzymes evoked above, as well as antioxidant defenses (Regoli et al. in Amiard-Triquet et al. 2011) and stress proteins

(Mouneyrac and Roméo in Amiard-Triquet et al. 2011). These defense mechanisms have a positive impact on the health of biota, allowing the survival of organisms in a degraded environment. In highly contaminated zones, many plant and animal species are indeed able to cope with the presence of potentially toxic substances (Amiard-Triquet et al. 2011). On the other hand, development of tolerance through physiological acclimation and genetic adaptation can induce energy and fitness costs (Mouneyrac et al. in Amiard-Triquet et al. 2011).

Biomarkers of damage reveal more or less severe biological impairments, potentially responsible for detrimental effects on reproduction or even survival. The importance of toxic effects depending on the degree of environmental contamination is quantified using a dose–effect relationship. The lowest doses do not induce any noxious effect, but with increasing doses biological impairments are progressively enhanced. The theoretical dose–effect relationship is depicted in Figure 1.2 for different levels of biological organization. The curve is limited to the domain of low doses to show the first observed effects or initial effects. At the molecular level, the initial effect is observed at a dose X_1 that is lower than the dose X_2 able to induce a cellular effect, this in turn being lower than X_3, acting at the tissue level. The same argument can be expanded to the level of organs, individuals, populations, etc. This scheme highlights that the lower the level of biological organization, the more sensitive the biological response will be. The rationale for this is quite evident: if only a few molecules have suffered a toxicant effect, cell functioning will not be significantly disturbed; if only a few cells are no longer functional within a whole organ, the function of this organ will still be efficient.

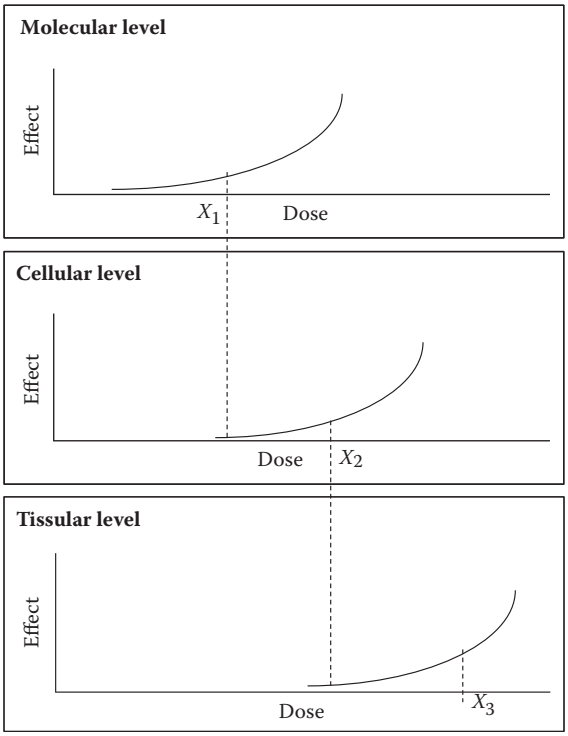

FIGURE 1.2
Biomarkers of damage: progression of the dose–effect relationship according to the level of biological organization.

Because responses of biomarkers of damage at the lowest levels of biological organization are so sensitive, they would appear to have the potential to be particularly useful in a management scheme to prevent any pollution effect. However, because organisms have very efficient mechanisms of regulation and repair, the use of such low level biomarkers brings with it a serious risk of a false positive if they are used as a warning signal for impairments at the level of communities or ecosystems. This is even more true for biomarkers of defense since this type of biological response shows that the organisms are coping actively with environmental degradation.

To put more ECO into ECOtoxicology, Chapman (2002) recommends the use of biological models more representative of the communities or ecosystems under examination than organisms classically used in biomonitoring programs or laboratory tests. It is generally admitted that protecting the most sensitive species within an ecosystem results in the protection of the whole community. This notion of susceptibility is not so simple. Reproduction and development of juveniles are commonly used as endpoints when assessing interspecific susceptibility to chronic toxicity, because these life traits are considered equally relevant in all species. This hypothesis was tested in two nematode species exposed to copper (Kammenga and Riksen 1996). Despite juvenile survival, duration of juvenile and reproduction periods, and daily reproduction rate being more affected in one species, fitness (which was defined by these authors as the population growth rate) was identically reduced in both species.

Species most commonly used as biological models in ecotoxicology are representative of the water column, whereas it is well established that sediments and soils are the main stores for a large majority of contaminants entering the environment. The choice of the most relevant species for the determination of biomarkers will be discussed in Chapter 7, considering the different objectives of conservation programs: ecosystem functioning, biodiversity integrity, survival of charismatic species, etc.

Responses to pollutants at different levels of biological organization are depicted in Figure 1.3 in the case of fish, considering the latency between exposure and the occurrence of the effect on the *X* axis, and the degree of ecological relevance on the *Y* axis. Molecular effects that are the most sensitive (Figure 1.3) are also the most precocious. On the other hand, they are mainly toxicological tools for which ecological relevance is poor. In contrast, population or community responses are obviously relevant to assess the "good ecological status" or "ecological integrity" of water masses [United States' Clean Water Act (CWA), 1972; European Community Water Framework Directive (WFD), 2000], but effects at these levels become significant only after severe environmental degradation has already occurred, thus leading to expensive remediation processes.

An extreme case provides a striking illustration of the magnitude of remediation problems: the experiences of the Minamata Bay project in Japan (Hosokawa 1993). A chemical factory released mercury into this bay from 1932 to 1968, leading to the death of 900 people among more than 2000 affected patients as a result of seafood contamination. The remediation project commenced in 1977 and was completed in 1990 after 1.5 million m^3 of Hg-contaminated sediment had been treated by careful dredging and confined reclamation at a total cost of 48,500 millions yen (equivalent to 650 millions).

Is it possible to reconcile the benefits of biochemical markers and ecological responses? It may be seen in Figure 1.3 that processes involved in reproduction include a set of responses from the molecular level leading to consequences of reproductive success on the sustainability of populations in ecosystems impacted by anthropogenic activities. Although it is excessive to consider that the pursuit of toxicological endpoints other than those concerned with reproduction is likely to be a wasted effort (Tannenbaum 2005), it is

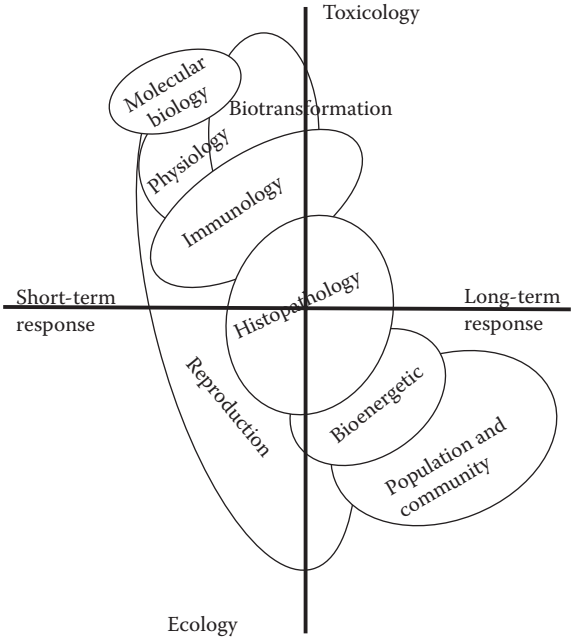

FIGURE 1.3
Latency between exposure of fish to pollutants and the occurrence of effects at different levels of biological organization. (After Adams, S.M. et al., *Mar. Environ. Res.*, 28, 459–464, 1989.)

evident that reproductive success is key for environmental conservation. The impairments at infra-individual and individual levels that can most probably affect the success of reproduction are depicted in Figure 1.4. These include endocrine disruption (Chapters 8 and 9), behavioral changes (Chapter 10), energy disturbances (Chapters 11 and 12), and genetic responses either adaptive or detrimental (Chapters 13 and 14).

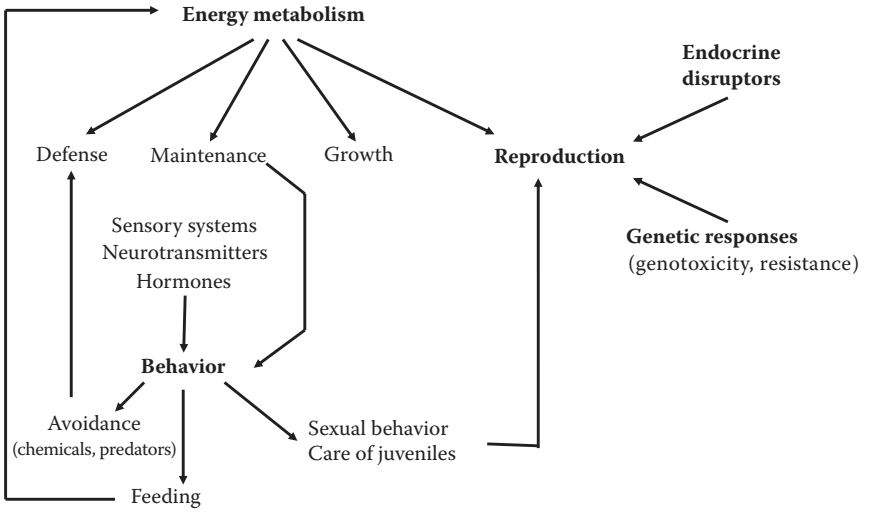

FIGURE 1.4
Linkage between effects of contaminants from molecular to population levels via the success of reproduction.

The problem of endocrine disruption was first realized because of the disastrous ecotoxicological effect of tributyltin (TBT), a compound used in antifouling paints. TBT-mediated imposex (for details, see Chapter 9) has been observed in more than 195 species of prosobranch gastropods worldwide (Sternberg et al. 2010). Subsequent population depletion of such gastropods has been observed in harbors and marinas where many individual snails were presenting morphological symptoms of imposex. In the case of the dogwhelk *Nucella lapillus*, population-level effects on other species (barnacles, fucoid seaweeds, hermit crabs) belonging to the same ecological community would be attributable to such a population drop in the affected gastropods (Bryan and Gibbs 1991).

Endocrine glands and the hormones they secrete are not only indispensable to the success of reproduction but are also involved in the development of organisms, their growth, and their behavior. However, most scientific research, particularly in fish, focuses on interactions between pollutants and male and female sexual hormones (Chapters 8 and 9). A peculiar topic of concern is that the effects of endocrine disruptors on reproduction are typically subtle, occurring at low doses, in the absence of any other appearance of toxicity. The spatial distribution of endocrine-disrupting chemicals, particularly steroid estrogens and nonylphenols, is related to the discharge of domestic and industrial wastewaters everywhere in the world (Jugan et al. 2009; Bertin et al. 2011; Gong et al. 2011; Tetreault et al. 2011). The presence of intersex (male gonads invaded with oocytes) individuals is increasingly documented in bivalves and fish. Natural or xenoestrogens could be a contributory factor in the induction of intersex (Baroiller and D'Cotta 2001; Langston et al. 2007). However, it is still unclear if intersex can have consequences on the production of progeny (Chapters 8 and 9).

A wide variety of anthropogenic, waterborne contaminants can also affect the hypothalamic–pituitary–thyroid axis and its role in development and reproduction as recently reviewed in teleost fish and amphibians (Blanton and Specker 2007; Carr and Patiño 2011). Impairment of thyroid functioning can influence behavior as neurotoxic effects such as the inhibition of neurotransmitters (AChE, serotonin) have also been observed (Figure 1.4). Many aspects of behavior can be affected (Dell'Omo 2002; Amiard-Triquet 2009; Hellou 2011): avoidance of predators or contaminated sediment or other habitat, contributing to the defense and survival of organisms; location of sexual partners and care of juveniles indispensable to reproductive success; feeding behavior and prey capture important for acquiring energy. Thus, behavioral ecotoxicology is potentially useful to link biochemical impairments to population effects (Chapter 10).

The success of reproduction is clearly linked to the relative energy allocation of an organism to defense against exposure to chemical stressors, basal metabolism, growth, and reproduction. Organisms obtain their energy from ingested food. For predators, the impairment of foraging activity can lead to a shift toward easily accessible food such as detritus, the energy value of which may be lower. Chemical contaminants can also influence food assimilation through the impairment of digestive enzyme activity. Lastly, prey species can be susceptible to environmental contamination, thus leading to decreased food availability for predators (Chapter 11).

Energy analysis can reveal a disequilibrium in energy balance associated with toxic or more general stress. Different energy parameters can be used as biomarkers of pollutant effects (Chapter 12). These parameters can be linked to macroscopic criteria representative of maintenance and growth (condition indices, size, or biomass increase, etc.) or reproduction (gonadosomatic index, egg production, offspring number, etc.). For ecological risk assessment, it is necessary to determine to what extent populations may be affected when such adverse effects are revealed (loss of their ecosystem function or even local extinction). Models that can allow extrapolation from individual- and suborganismal-level responses

to the population level have been reviewed (Maltby et al. 2001). Among those, dynamic energy budget models combined with demographic models have been well developed (Charles et al. 2009).

Exposure to chemicals can lead to DNA damage (Figure 1.5), the consequences of which may be limited by DNA repair (Peterson and Côté 2004). Mutations frequently have toxic effects, including carcinogenesis, and when affecting germinal tissues, they are inheritable and can also affect future generations, provided that the offspring are viable and able to survive and reproduce. In fact, impairments of germinal cells often result in embryo lethality or early death of the progeny. From an ecological point of view, it is questionable if these precocious deaths can impact the fate of populations (Manahan 2003; Newman and Clements 2008). In some cases, mutations can confer a selective advantage leading to the selection of resistant genotypes. Biomarkers of exposure to genotoxic pollutants are reviewed in Chapter 13, and Vasseur et al. explore the relationships between genotoxicity and population effects.

Chronic exposure to chemicals can exert a selection pressure leading to the presence of resistant genotypes in organisms living in impacted areas. The acquisition of tolerance is particularly well documented for pesticide-exposed insects (Hemingway et al. 2004), but other classes of contaminants (metals, PAHs, PCBs) can be responsible for the predominance of resistant genetic patterns in bacteria (Nies 1999), plants (Frérot et al. in Amiard-Triquet et al. 2011), invertebrates (Nevo et al. 1984), and vertebrates (Athrey et al.

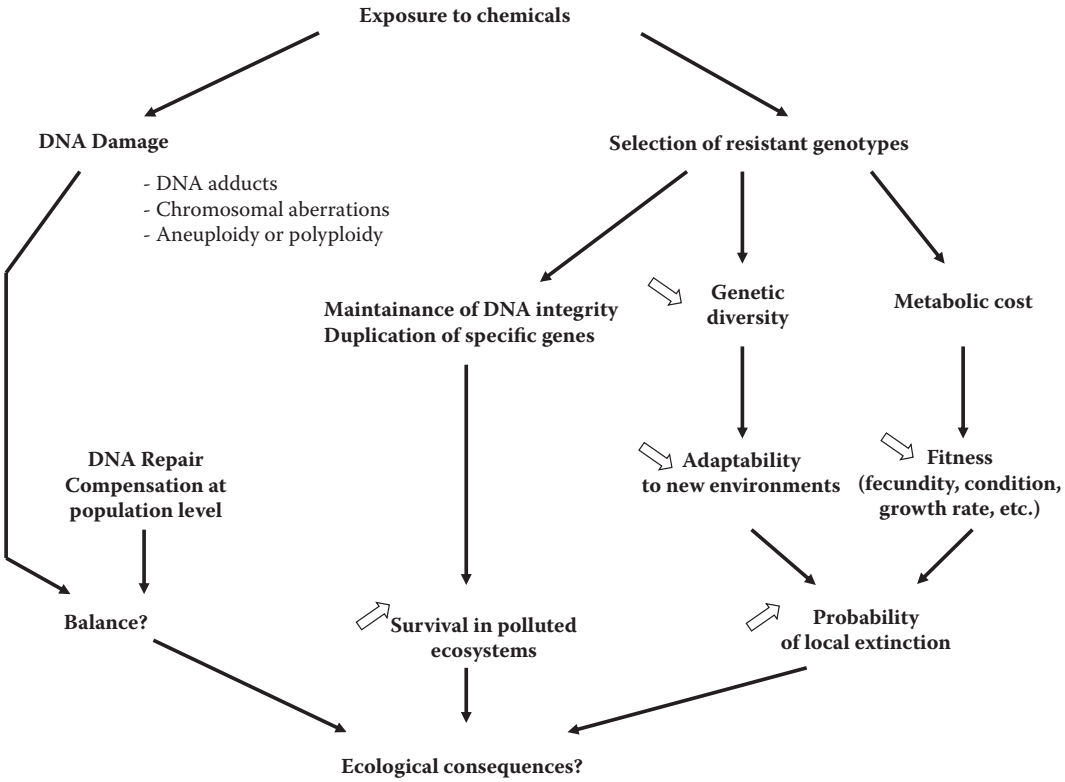

FIGURE 1.5
Genetic responses to chemical exposure: DNA damage versus selection of resistant genotypes.

2007; Romeo and Wirgin in Amiard-Triquet et al. 2011). In contaminated areas, an increased frequency of resistant genotypes has often been reported, allowing the maintenance of DNA integrity associated with the duplication of specific genes (Figure 1.5). However, negative consequences of being resistant may be observed, such as decreased fitness and decreased adaptability to new environments or stressors, thus increasing the probability of local extinction (Chapter 14).

Biomarkers are available as crucial tools in ecotoxicology, because they can be used as early warning signals of environmental change before the onset of irreversible damage at the population level. Syntheses published at the turn of the century (Lagadic et al. 1997, 1998; Garrigues et al. 2001) suggested that scientists were then ready to transfer the methodology of biomarkers to end users in charge of environmental biomonitoring. A decade later, certain biomarkers are used to assess the health status of aquatic environments in different parts of the world (Chapter 15). However, this use is generally limited to a relatively small number of more or less specific biomarkers, the worst counterexample being the WFD—a very important regulation aiming at the protection of aquatic environments from the river source to the seashore—which totally ignores the use of biomarkers despite the efforts of European scientists to demonstrate the relevance of biomarkers as tools for the implementation of the WFD (Allan et al. 2006; Hagger et al. 2008; Sanchez and Porcher 2009). Independently of regulatory frameworks, many important studies have demonstrated "the usefulness of applying a large array of various combined biomarkers at different levels of biological organization, in assessing the toxic effects of a mixture of pollutants in a natural aquatic environment" (Huadi River, a tributary of the Pearl River, China) (He et al. 2011). In the Bay of Cadiz, biomarkers determined in caged clams *Ruditapes philippinarum* allowed assessment of chemical exposure and sediment quality (Ramos-Gómez et al. 2011). In the Río Champotón (southwestern Mexico), a set of biomarkers determined in a native fish *Astyanax aeneus* was shown to be a sensitive and effective tool for identifying periods of environmental conditions adverse to fish health (Trujillo-Jiménez et al. 2011).

Several problems contributing to limit the use of biomarkers have been recognized: the problem of confounding factors (e.g., Thain et al. 2008; Martínez-Gómez et al. 2010), the question of a reference site, and the lack of ecological relevance (Forbes et al. 2006). The problem of confounding factors was well conceptualized by Cairns (1992). When a biological parameter is highly fluctuating, the occurrence of a stress may be concealed by natural fluctuations. On the other hand, when background values are relatively stable, any change due to contamination factors is easily revealed (Figure 1.6). As already mentioned by Kalman et al. (2010), "The question of confounding factors is well mastered in biomonitoring programs based on the determination of contaminants in the tissues of bioaccumulators such as the bivalves used in the 'Mussel Watch'–type programs." The literature indicates that the same natural factors are at work in the case of biomarkers (Thain et al. 2008). Consequently, in the objective of using a peculiar species as a model for the determination of biomarkers, it is still indispensable to determine the natural fluctuations, as exemplified for worms (Kalman et al. 2010), bivalves (Burgeot et al. 2010; Fossi Tankoua et al. 2011), and fish (Sanchez et al. 2008). Temporal surveys provide significant advantages over spot sampling techniques, allowing the assessment of pollution trends responsible for population changes while providing data on background levels that would be of great use in case of a future accident, as often experienced for oil spills (Martínez-Gómez et al. 2010).

For many aspects of environmental monitoring, our present state of knowledge and the insufficiency of background data available mean that the use of a reference site for

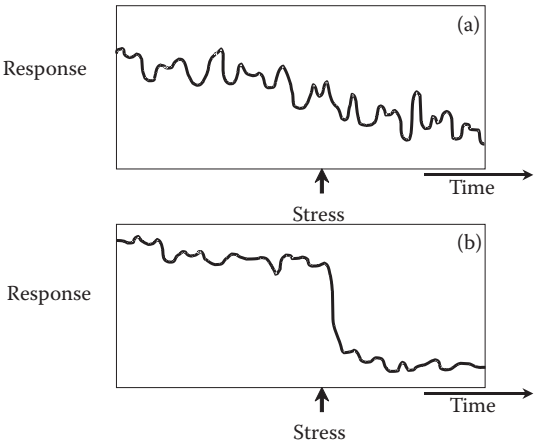

FIGURE 1.6
Relative importance of natural fluctuations of a biomarker response compared to stress-induced response. (a) Highly variable background masking stress response. (b) Background relatively stable allowing significant variation due to stress. (After Cairns, J. Jr., *Ecotoxicology*, 1, 3–16, 1992.)

comparison is essential. However, to date, with the worldwide dispersion of contaminants evoked above, pristine areas have disappeared and, at best, reference sites can be chosen in only a few places that remain comparatively clean. To choose a reference site, geographical proximity and similarity in terms of temperature, granulometry, and organic content of sediment, salinity regime (in estuaries), etc., are mandatory to mitigate the importance of confounding factors. This is not an easy task, as described, for instance, in estuaries (Amiard-Triquet and Rainbow 2009). Potential reference estuaries with low perceived anthropogenic pressure are generally small, whereas the human activities responsible for the presence of many chemicals in the environment have historically developed on the banks of larger main watercourses. This does provide a potential problem when trying to eliminate comparative differences resulting from hydrodynamic differences between the estuaries under comparison. Even in the less fluctuating conditions of a freshwater biomonitoring program, the interpretation of fish biomarker results is strongly influenced by the selected reference system (Sanchez et al. 2010).

The addition of more than one reference site into any comparative study, however superficially attractive, has significant resource implications. Associated with the need for temporal surveys instead of spot sampling techniques and the development of the need to analyze a battery of biomarkers (Chapter 2), methodology involving biomarkers is not always as initially claimed: sensitive, simple, and cost-effective. Even despite this complexification, the biomarker methodology to be proposed to end users—although efficient in assessing chemical exposure, sediment quality, and the toxic effects of mixed pollutants—still fails at predicting chemical risk at supra-individual levels (Forbes et al. 2006). The development of an integrated indicator framework using biological effect techniques remains key to improve the risk assessment of contaminants in aquatic ecosystems (Thain et al. 2008).

Since pioneering papers (Atrill and Depledge 1997; Clements 2000) underlined the importance of targeting links between levels of biological integration, certain research groups have focused their attention on the cascading effects of interrelated biomarkers that can be linked to important biological processes and for which changes can be

interpreted (Amiard-Triquet and Rainbow 2009; Ankley et al. 2010; Taylor and Maher 2010; Mouneyrac and Amiard-Triquet, accepted). Ecologically relevant biomarkers such as lysosomal integrity (Chapter 5), immunotoxicity (Chapter 6), endocrine disruption (Chapters 8 and 9), behavior (Chapter 10), energy metabolism (Chapters 11, 12), and genomic biomarkers (Chapters 13, 14) appear to be promising candidates to fill the gap existing between suborganismal and organismal responses to stress and effects occurring at higher levels of biological organization.

The main objective of the present book is to review biomarker research that examines the effects of contaminants using an integrative approach. In order to improve the predictive value of biomarkers, special attention will be devoted to biological responses that can be observed at infra-individual or individual levels (early and sensitive warning signals) but have a serious potential to reveal threats at supra-individual levels (population, community, ecosystem). For each category of biomarkers (biochemical, physiological, behavioral, etc.), their usefulness for predictive (e.g., effects of different nanoparticles in aquatic organisms, Koelher et al. 2008; Li et al. 2009; Galloway et al. 2010; Ringwood et al. 2010; Tedesco et al. 2010; Buffet et al. 2011) or retrospective (e.g., adverse effects of pharmaceuticals in wild fish; Sanchez et al. 2011) risk assessment of emerging contaminants will be considered. The final aim is to contribute to the search for a conceptual framework to support the assessment of the health status of aquatic ecosystems.

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2

History of Biomarkers

Michèle Roméo and Laure Giambérini

CONTENTS

2.1 Context

Although knowledge of the existence of a link between biological dysfunction and the environment is very old, as testified by writings dating from more than 2000 years ago (Hippocrates, translated by Littré 1861), serious consideration of pollution by both society and scientists is a more recent phenomenon. Rachel Carson, fighting against the unreasonable use of organochlorine pesticides and their effects on living organisms, in her book *Silent Spring* (Carson 1962), can be considered a pioneer for ecotoxicological studies. After a period when the effects of the dispersion of chemical compounds into the environment tended to be evaluated *a posteriori* and possibly corrected, a will to carry out evaluations *a priori* was essential in the last quarter of the twentieth century. Until the end of the 1980s, monitoring of the environment was based on conventional chemical methods of variable significance (chromatography, spectrophotometry, electrochemistry, radiochemistry, etc.), generally leading to the measurement of concentrations of chemical substances considered to be dangerous, in water, sediments, and organisms living in coastal ecosystems.

Although such chemical analyses are essential to identify concentration trends of contaminants (increase, plateau, or reduction) in the environment, they do not provide information about the real impact of the pollutant on its final target—the living organism. It is apparent then that this physicochemical assessment is insufficient to evaluate the health of a complex medium, with a mixture of contaminants potentially leading to the phenomena of synergy and antagonism. The concept of biological monitoring, based on the study of the biological response of organisms to pollutants, termed biomarkers, is today well established. The characterization of these biomarkers can constitute an early warning system before the further deterioration of the structure and function of an organism, and particularly before all the population or the ecosystem is disturbed. This concept is not new: it is the principle of diagnosis in human medicine, founded on the detection of symptoms likely to reveal a disease (Lafaurie et al. 1992).

2.2 Definition

In the past nearly 30 years, several definitions of biological markers have been published. The historical development of the biomarker approach has been closely related to advances in medicine and biology of vertebrates [National Research Council (NRC) 1987]. Biological markers were classified as exposure, effect, and susceptibility biomarkers. Moreover, in the publications of the NRC (1987, 1989), the authors highlighted that biological markers can be simultaneously used for biological monitoring and for monitoring of health. According to McCarthy and Shugart (1990), "biological markers are measurements at the molecular, biochemical, or cellular level in either wild populations from contaminated habitats or in organisms experimentally exposed to pollutants that indicate that the organism has been exposed to toxic chemicals, and the magnitude of the organism's response to the contaminant."

The definition was generalized by Depledge (1994): a biomarker is "a biochemical, cellular, physiological or behavioral change which can be measured in body tissues or fluids or at the level of the whole organism that reveals the exposure at/or the effects of one or more chemical pollutants." In September 1994, the journal *Ecotoxicology* presented four reviews on the role of the biomarkers in environmental assessment, as carried out with invertebrates (Depledge and Fossi 1994), vertebrates (Peakall and Walker 1994), terrestrial plants (Ernst and Peterson 1994), and populations and communities of invertebrates (Lagadic et al. 1994). These articles were required by the European Foundation for Science (ESF) to understand to what extent biomarkers could be used to evaluate environmental damage and to formulate possible rules to control any such damage.

Finally, Van Gestel and Van Brummelen (1996) attempted a redefinition of the terms biomarkers, bioindicators, and ecological indicators, by calling on previous work published in *Ecotoxicology* in 1994 when Lagadic et al. (1994) made a clear distinction between biomarkers and bioindicators and restricted the use of the term "biomarker" to the sublethal biochemical changes resulting from individual exposure to xenobiotics. However, this reductionist definition was not generally accepted (Van der Oost et al. 2005; Allan et al. 2006), with many scientists voicing their concern about not neglecting responses (e.g., physiological, behavioral) that could be used in risk assessments involving a change in scale of biological organization from the individual to the population. According to Van Gestel and Van Brummelen (1996), a biomarker is defined as any biological response to an environmental chemical contaminant at the infra-individual level, measured in an

organism or its products (urine, feces, hair, feathers, etc.), indicating a change compared to the normal state and which cannot be detected in a healthy organism. The term bioindicator should be restricted to an organism providing information on the environmental conditions of its habitat by its presence or its absence or its behavior. The concept of specific biomarkers (responding to metal pollutants, or to organics or to any defined pollutant) led to the definition of damage and defense biomarkers put forward by De Lafontaine et al. (2000). From the 1970s, great developments in biochemistry and molecular toxicology made it possible to progress quickly in our knowledge of the mechanisms of the toxicity of xenobiotics, mainly with mammalian models. Thereafter, significant specific biochemical effects were highlighted in species exposed to some contaminants, particularly in birds, fish, and mollusks considered as being of ecological interest. The majority of the examples in this chapter concern the aquatic environment, particularly the marine environment, which is the final receptacle of chemical pollutants.

Well-known biomarkers, which have been recognized in laboratory and environmental studies, have been called "core biomarkers" (Pampanin et al. 2006). Such core biomarkers include the stability of the lysosomal membrane (measured by the neutral red retention time), inhibition of acetylcholinesterase (AChE) activity, metallothionein (MT) concentration, ethoxyresorufin *O*-deethylase (EROD), and the fluorescent metabolites of the bile [fluorescent aromatic compound (FACs)].

2.3 Defense Biomarkers

2.3.1 Ethoxyresorufin *O*-Deethylase

Payne and Penrose (1975) were among the first to report elevated cytochrome P450–dependent monoxygenase activity in fish from petroleum-contaminated areas. The first biomarker that gained international recognition was consequently the enzymatic activity of EROD, an isoenzyme cytochrome P4501A termed as CYP1A. EROD belongs to the group of CYP enzymes that are the main enzymes responsible for the metabolism of certain endogenous compounds (hormonal and membrane steroids, biliary acids, vitamin D, fatty acids, prostaglandins, and pheromones) and nonpolar xenobiotics, including the metabolism of many environmental toxic chemicals and carcinogens (Nebert 1994). CYPs are enzymes referred to as mixed function oxidases (MFOs) (Di Giulio et al. 1995). Klingenberg (1958) and Garfinkel (1958) described successively a pigment present in the microsomal fraction from mammalian liver, which, in its reduced form, fixes carbon monoxide and absorbs at 450 nm. The denomination "P450 cytochrome" was proposed by Omura and Sato (1964), who showed that this pigment is a hemoprotein with molecular mass ranging from 43 to 60 kDa. For the first time, Estabrook et al. (1963) demonstrated the involvement of this hemoprotein in a reaction of monoxidation: the hydroxylation of 17α-hydroxyprogesterone. CYPs are found to be associated with membranes in the endoplasmic reticulum or mitochondria of different tissues: liver, lung, kidney, intestine, etc. (Stegeman and Hahn 1994). They catalyze the oxidation of a substrate RH (an organic compound that becomes hydroxylated) by inserting one atom of molecular oxygen, whereas the second atom is reduced to water following the equation:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + NADP^+ + H_2O$$

This reaction constitutes the first phase (phase I) of the biotransformation of organic compounds that causes hydrophobic compounds to become more water soluble.

The *de novo* synthesis of P450 proteins by organisms termed as "induction" leads to increased enzymatic activity. Induction has been well known for 40 years in humans and other mammals, more recently in fish and plants, and of late in invertebrates (Stegeman and Hahn 1994). The induction of cytochrome P450 isoenzymes responds to exposure to xenobiotics by way of a selective, receptor-mediated stimulation of the CYP1A gene transcription rate, resulting in increased levels of specific mRNA, new synthesis of cytochrome P450 isoenzymes, and an increase in their catalytic activities (e.g., EROD for CYP1A). The receptor that mediates the regulation of the CYP1A gene expression is known as the AH (aryl hydrocarbon) receptor (AHR) (Poland and Glover 1975; Guengerich 1993). Studies have demonstrated that activation of the AHR pathway is necessary for benzo[*a*]pyrene (B[*a*]P)-induced hepatic carcinogenicity in mice (Shimizu et al. 2000), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorobiphenyl (PCB) induced early life stage toxicities in fish (Antkiewicz et al. 2006). The functioning of the AHR pathway in fishes is almost identical to that in mammals, except that fish have two or more forms of AHR (AHR1 and AHR2) due to genome duplication events (Hahn 2002). After diffusing into the cell, the xenobiotic binds to a protein complex in the cytoplasm consisting of AHR, a dimer of heat-shock protein 90 (Hsp90), p23, and ZAP2 (also known as ARA9 and AIP) (Figure 2.1). Upon ligand binding, ZAP2 is released, exposing the nuclear localization signal on AHR and

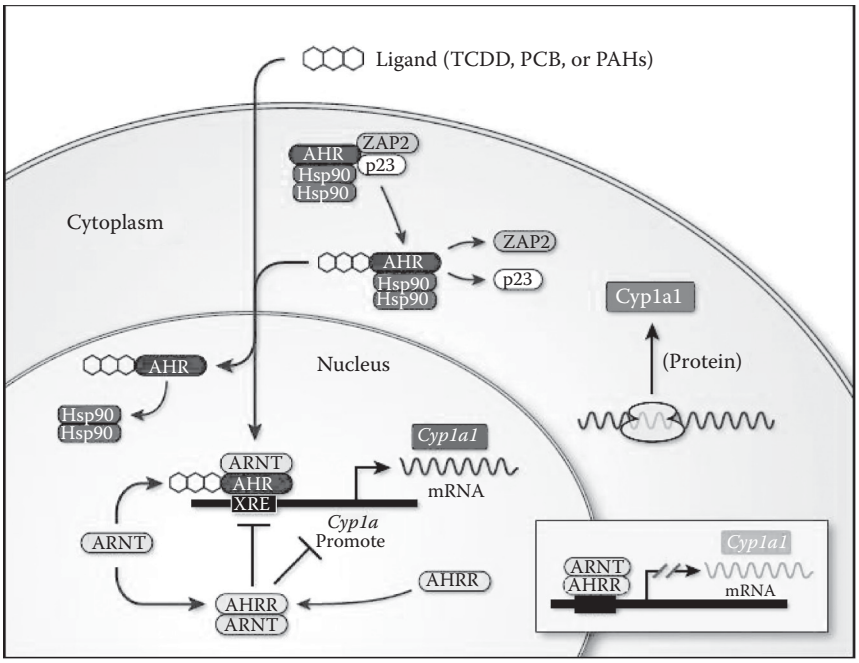

FIGURE 2.1

Functioning of the AHR (aryl hydrocarbon receptor) pathway in fishes. After diffusing into the cell, the xenobiotic binds to a protein complex in the cytoplasm consisting of AHR, Hsp90, p23, and ZAP2. Upon ligand binding, ZAP2 is released leading to translocation of AHR from the cytoplasm to the nucleus. Within the nucleus, Hsp90s are released, and AHR heterodimerizes with the Aryl Receptor Nuclear Translocator (ARNT). The AHR–ARNT complex then binds to multiple enhancer elements in the promoter region of responsive genes in the AHR battery such as CYP1A. (From Figure 8.2 of Roméo, M., Wirgin, I.I., in C. Amiard-Triquet, P.S. Rainbow, and M. Roméo, *Tolerance to Environmental Contaminants*, CRC Press, Boca Raton, 175–208, 2011. With permission.)

leading to translocation of AHR from the cytoplasm to the nucleus. Within the nucleus, Hsp90s are released, and AHR heterodimerizes with another protein, the Aryl Receptor Nuclear Translocator (ARNT). The AHR–ARNT complex then binds to multiple enhancer elements in the promoter region of responsive genes in the AHR battery such as CYP1A.

The P450 enzymes, involved in the detoxification of xenobiotics, are slightly expressed under normal physiological conditions, but are on the other hand strongly inducible: their content or their activity is increased in response to one or more exogenic molecules. The biological advantage of this induction process by xenobiotics is generally to amplify their metabolic degradation. Nelson regularly publishes a review of P450 cytochromes according to their families and subfamilies (drnelson.uthsc.edu/CytochromeP450.html). As of February 2009, more than 8100 distinct CYP gene sequences have already been known. The nomenclature used for cytochrome P450s is based on sequence homology (Nebert and Nelson 1991): two cytochrome P450s belong to the same family when their peptide sequence presents more than 45% amino acid homology and to the same subfamily if the homology is higher than 55%. The abbreviation CYP (cytochrome P450 gene) is completed with a number representing the family, then a letter indicating the subfamily (e.g., CYP4A), and a last number when there are several genes within the same subfamily (e.g., CYP4A1, CYP4A2). Conventionally, genes are written in italics *CYP1A1* (Goksøyr and Förlin 1992), whereas mRNA and proteins are in capitals. Nelson (1998) has developed a classification scheme where CYP families are classified into CLANS, that is, clusters of higher order groupings of P450 families.

They are ubiquitous proteins, the presence of which was demonstrated in plants and animals, from bacteria to mammals. P4501A1 enzymes (in particular, EROD measured in fish) may be induced by compounds sterically analogous to dioxin such as aromatic hydrocarbons, polychlorinated biphenyls, and polychloroazobenzenes. The first work on EROD and other P450 enzymes as biomarkers was completed on freshwater and marine fish livers (Addison 1984; Addison and Payne 1987; Flammarion et al. 1998). Polycyclic aromatic hydrocarbons (PAHs) induce P4501A1 in all fish considered by different authors from agnathans to teleosts and selachians (Stegeman 1987; Andersson and Nilsson 1989).

CYP1As are induced by PAHs, coplanar PCBs, polychlorinated dibenzodioxins, and polychlorinated dibenzofurans (Goksøyr and Förlin 1992), which are pollutants of the 3-methylcholanthrene type and are now considered AH receptor agonists. Three enzyme activities, EROD, ethoxycoumarin *O*-deethylase, and arylhydrocarbon (B[*a*]P) hydroxylase are largely specific in their response to these compounds. Many PAHs are both inducers and substrates for CYP1A. In contrast, coplanar PCBs, although often good inducers, are frequently poor substrates for CYP1A (Di Giulio et al. 1995). In their review, Goksøyr and Förlin (1992) reported that CYP2B is induced by coplanar PCBs (phenobarbital type), CYP3A by endogenous steroids, and CYP4A by endogenous fatty acids and xenobiotics such as phthalates and peroxisome proliferators (Simpson 1997). Therefore, members of the cytochrome P450 family of monoxygenases can metabolize and often produce more toxic forms from (see below) a wide variety of endogenous molecules and xenobiotics.

In contrast to fish, the presence of the AH receptor is not confirmed in mollusks. The cytochrome P450 pathway in PAH metabolism in mussels is low compared to the radical manner which leads to the formation of quinones. However, the existence of a CYP1A-like gene in mussels (Wootton et al. 1995) justifies research into the mechanisms of activation and detoxification already identified in fish. The capacity to metabolize *in vitro* B[*a*]P into derived diol, quinone, and phenol was demonstrated in the mussel *Mytilus galloprovincialis* (Michel et al. 1993). The activity of B[*a*]P hydroxylase BPH, measured in the digestive gland of this mussel (measurement based on the production of phenol metabolites resulting from

B[*a*]P oxidation), proved to be a biomarker of exposure to PAHs (Akcha et al. 2000). In some cases, the biotransformation can induce processes of carcinogenesis, mutagenesis, and toxicity. For example, B[*a*]P is metabolized (7,8-epoxidation, then 9,10-epoxidation) into a mutagenic compound, the (+)-anti-B[*a*]P, 7*R*,8*S*-diol-9S, 10*R*-epoxide, which is able to bind in a covalent manner to DNA and leads to the formation of adducts (Vermeulen 1996; Akcha et al. 1999).

2.3.2 Fluorescent Aromatic Compounds in Fish Bile

The exposure of fish to crude oils containing PAHs causes an increase in FACs in the bile (Aas et al. 2000; Gagnon and Holdway 2000). When the exposure takes place through the food chain, PAHs are absorbed, transported to the liver where they are converted into more water-soluble metabolites, and are excreted in the bile (Varanasi et al. 1995; Lee 2002). Laboratory studies show that the depuration period after exposure lasts several weeks, suggesting that an increased concentration in FACs in bile reflects a relatively recent exposure to PAHs (Huggett et al. 2003). Crude oils with PAHs with two to three rings are very different in their FACs in bile compared to pyrogenic hydrocarbons with four to six nonsubstituted rings. This is why it is difficult to link the induction of CYP1A and the increased concentrations of FACs in the bile to a specific source of PAHs. However, the concentration of FACs in the bile constitutes a fast and practical tool that clearly shows the extent of exposure to PAHs in the framework of biomonitoring: they thus constitute a "relevant" biomarker (Lehtonen et al. 2006).

2.3.3 Phase II Enzymes

Conjugation intervenes in the metabolism of xenobiotics, either following the reactions of oxidation (phase I), or directly on molecules bearing hydroxylated, thiol, or carboxylic groups. These reactions, also called phase II reactions, are catalyzed by membrane or cytosolic enzymes functioning with various cofactors (glutathione, sulfates, glucuronic acid). The enzymes responsible for these conjugations are glutathione *S*-transferases (GSTs), UDP-glucuronosyl-transferases (UDPGTs), and sulfotransferases. The activities of phase II enzymes are lower in fish (Gregus et al. 1983) than in higher vertebrates. In the fish *Platycephalus bassensis*, exposed to a mixture of PCBs, UDPGT activities significantly increase as do cytochrome P450 enzymes (Brumley et al. 1995), whereas the exposure of trout *Salmo gairdneri* to various polychlorinated phenols causes a reduction in UDPGT activities (Castren and Oikari 1987). GSTs are enzymes whose activity is used as a biomarker of organic substance exposure, especially in mollusks, where EROD activity is not routinely measured (Cajaraville et al. 2000). GSTs represent an important enzyme family whose function is to combine reduced glutathione (GSH) with electrophilic compounds by formation of a thioether bridge (Foureman 1989). The products are then metabolized in mercapturates that are excreted in the bile or the urine. GST activity increases in exposed organisms according to the xenobiotic concentration in the medium.

In fish, contradictory results have been reported (Van Veld and Lee 1988). However, several authors have shown that glutathione transferases are involved in the detoxification of many chemical pollutants: hydrocarbons, organochlorine insecticides, and PCBs (Monod et al. 1988; George 1994). In mollusks, GST activity is used with more success than in fish as a biomarker of exposure to these substances (in the marine environment: Fitzpatrick et al. 1997; Hoarau et al. 2001; and for freshwater bodies: Boryslawskyj et al. 1988; Robillard et al. 2003). GSTs play an additional role in the detoxification process, being used as transporting

molecules that increase the bioavailability of lipophilic compounds to the phase I enzymes [such as mixed function oxygenases (MFOs)]. They therefore reduce, by covalent linkage to electrophilic compounds, the probability of these compounds binding to other cellular macromolecules such as DNA (Van Veld et al. 1987).

2.3.4 Phase III Enzymes

Surprisingly, after phase II, it was generally considered that the xenobiotics were "detoxified" and no longer considered. However, accumulation of the metabolites that may result in cell injury and their excretion, occurring during phase III of biotransformation, is of particular importance (Damiens and Minier 2011). Phase III includes detoxification enzymes involved in the elimination from the cell of phase I and II products (metabolites) by transmembrane transport carried out by P-glycoproteins (PGPs) or by multidrug resistance–associated proteins (MRPs) (Gottesman and Pastan 1993). By now, it has been realized that transport systems are just as important as the previously known processes (Leslie et al. 2005; Cascorbi 2006). Phase III proteins, involved in the modulation of exit from the cell, are involved in key processes that result in the modulation of toxicological effects, and the multixenobiotic transport system is considered a system governing intracellular contaminant bioavailability. Membrane proteins MRPs are part of the large family of ABC (ATP binding cassette) transporters present in prokaryote and eukaryote cells. These ABC transporters have almost all the same architecture, with two binding domains of ATP located in the cytoplasm, and two hydrophobic regions inserted in the plasma membrane.

The first PGP was discovered in 1976 (Juliano and Ling 1976) in the context of resistance to multiple chemotherapy, and was named MDR (multidrug resistance protein). It transported a large number of compounds with different structures and modes of action—hence, the idea was presented that if different organisms live, grow, and reproduce in contaminated environments, they must have mechanisms allowing them to be resistant. Kurelec (1992) showed that resistance to many xenobiotics (multixenobiotic resistance MXR) has similarities with MDR. MXR proteins are found throughout the tree of life. Kurelec (1992) has reviewed MXR proteins in aquatic organisms. The wide taxonomic distribution of these proteins and their induction in the presence of xenobiotics show their importance in the nonspecific defense of organisms (Tutundjian and Minier 2002). How MXRs expel pollutants is not yet well known. Some models assume that removal is carried out by an enzyme called "flippase," which would capture the substrates at the inner leaflet of the membrane and translocate them to the outer leaflet (Tutundjian and Minier 2002). Minier et al. (1993) showed that mussels *Mytilus edulis* and *M. galloprovincialis* and oysters *Crassostrea gigas* express proteins immunologically similar to mammalian MDR proteins. Moreover, there is a relationship between their expression levels and the level of environmental contamination. Parallel to these studies, Kurelec et al. (1995) showed that the MXR system of the gastropod mollusk *Monodonta turbinata* could be induced by treatment with hydrocarbons.

Competition studies for transport increased our knowledge of the substrates involved. The possibility for *M. edulis* to expel pesticides such as triazines has been demonstrated (Minier and Moore 1998). Results have enabled the description of the phenomenon of resistance that is present in aquatic organisms and is expressed when they are exposed to compounds such as organochlorine pesticides, PCBs, and PAHs (Kurelec et al. 1995; Galgani et al. 1996; Eufemia and Epel 2000). There are also xenobiotics that inhibit MDR; they are called "chemosensitizers," and their presence induces an increase in concentrations of pollutants in the body with subsequent damage (Smital and Kurelec 1998).

2.3.5 Metallothioneins

MTs are nonenzymatic proteins with a low molecular weight (12–15 kDa), high cysteine content, heat stability, and no aromatic amino acids. The thiol groups (–SH) of cysteine residues enable MTs to bind particular trace metals. The first MT was found in equine renal cortex (Margoshes and Vallee 1957). MTs or MT-like proteins have since been reported in many vertebrates including many species of fish (reviewed by Hamilton and Mehrle 1986), and in aquatic invertebrates (reviewed by Amiard et al. 2006) such as echinoderms (Riek et al. 1999), mollusks (Amiard-Triquet et al. 1998; Bebianno and Langston 1998; Bebianno et al. 2003) and their larvae (Damiens et al. 2004), and crustaceans (Roesijadi 1992), but also in terrestrial invertebrates (Dallinger 1996). In aquatic species, MT concentrations were measured mainly in tissues involved in the uptake, storage, and excretion of metals such as gills, digestive glands, and kidneys, but also in muscular and nervous tissues. Fowler et al. (1987) defined three classes of MT according to the location of cysteine residues in the amino acid sequences. Class I includes MTs of vertebrates and MTs with a closely similar structure (mollusks, crustaceans). Class II includes MTs whose structure does not resemble that of class I (*Drosophila*, sea urchins, nematodes, fungi, cyanobacteria), and finally the third class includes the nonprotein MTs, synthesized from glutathione such as phytochelatins, present in plants.

Several reviews have synthesized the research completed mainly in aquatic species concerning the structure and the functions of MTs as well as the progress of assay techniques (Roesijadi 1992, 1996; Roméo et al. 1997; Cosson and Amiard 2000; Cosson 2000; Isani et al. 2000; Amiard et al. 2006). MTs whose behavior is related to the chemistry of thiol groups assume many biological functions and even if some remain under discussion, in general, authors agree on the participation of MTs in the homeostasis and detoxification of essential metals such as zinc and copper and in the detoxification of nonessential metals such as cadmium and mercury. Studies have also shown MT involvement in protection mechanisms against oxidative stress, apoptosis, and growth regulation of nervous cells (Cavaletto et al. 2002).

In vertebrates as well as in invertebrates, MT levels differ according to species and tissues. They are generally higher in the gills and digestive gland in mollusks (Baudrimont et al. 1997). The concentrations vary in different tissues not only according to the developmental stage, age, sex, size, and nutritional status of an organism, but also according to their gonadic development under hormonal control (Hamza-Chaffai et al. 1995, 1999; Leung and Furness 2001; Bebianno et al. 2003; Riggio et al. 2003; Leiniö and Lehtonen 2005). If the organism is exposed to a very high metal concentration, MT synthesis can be inhibited, as demonstrated by George et al. (1992).

MT synthesis is mainly induced by metals (essential or not) such as Cu, Zn, Cd, Hg, and Ag but also to a lesser extent by organic compounds such as some pesticides or antibiotics. The great variability of induction and the various abiotic or biotic factors influencing MT synthesis lead to contradictory results in the literature, which have been discussed in a review relating to the role of MTs in invertebrates and their use as biomarkers (Amiard et al. 2006).

For about the past 20 years, many studies carried out in laboratory conditions and *in situ* have shown the potential of increased concentrations in MTs for use as biomarkers of exposure to contaminant metals. Currently in ecotoxicological studies carried out in terrestrial and aquatic environments, their measurement may be integrated into a multibiomarker approach so *inter alia* mitigating for the presence of other inducers than metals.

2.3.6 Enzymatic and Nonenzymatic Antioxidant Defenses

In biological systems, reactive oxygen species (ROS) are continuously produced by several mechanisms involving exo- or endogenous compounds such as xenobiotics (Di Giulio et al. 1989; Livingstone et al. 1990; Winston and Di Giulio 1991). In aerobic organisms, they are part of basal cellular metabolism such as cellular respiration or phagocytosis activity (Cossu et al. 1997; Valavanidis et al. 2006). Their production is also a result of the activity of different oxidative enzymes such as tryptophan dioxygenase, xanthine oxidase, and cytochrome P450 reductase that produce superoxide anions, and guanyl cyclase and glucose oxidase, which are able to generate hydrogen peroxide.

Moreover, chemical pollutants are important producers of ROS. The xenobiotics known for their redox properties such as quinones, transition metals, diazoïc staining, bipyridyl herbicides, and nitric aromatic compounds induce the formation of superoxide radicals.

The imbalance in favor of ROS production instead of their neutralization by antioxidant systems corresponds to oxidative stress. At the cellular level, it results in the alteration and more particularly in the oxidation of components such as DNA, proteins, and lipids and in a total disturbance of the redox balance (e.g., ratios GSH/GSSG and $NADH/NAD^+$). Its cytotoxic effects are expressed by structural and functional perturbations such as enzymatic inhibition, protein damage, lipid peroxidation, inflammatory processes, and apoptosis (Figure 2.2).

During evolution, aerobic organisms have developed antioxidant defense mechanisms whose main function is to block off and to deactivate ROS. The extent of oxidative damage is directly related to the efficiency of antioxidant systems occurring in the different species. The systems are composed of a suite of cytosolic enzymes [mainly superoxide dismutases (SODs), peroxidases, catalases], reducing molecules of low molecular weight (glutathione, ascorbates, urates) and several liposoluble vitamins (α-tocophérol, β-carotene).

Among enzymatic antioxidant systems, SODs correspond to a metallo-enzyme family (containing Cu, Zn, Fe, or Mn) known to convert superoxide anion in hydrogen peroxide, H_2O_2. Among peroxidases, glutathione peroxidase (GPx), depending or not on selenium,

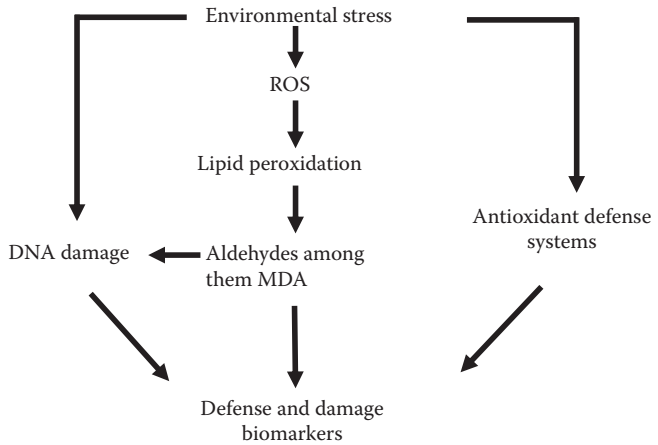

FIGURE 2.2
Environmental stress in organisms could generate ROS able to induce damage to membrane lipids and DNA molecules but also to antioxidant defenses. The cellular damage and the induction of defense systems could be used as defense or damage biomarkers.

uses reduced glutathione (GSH) to reduce different types of peroxides. Its enzymatic activity is related to that of glutathione reductase that generates GSH from the oxidized form of glutathione (GSSG). Catalases are hemoproteins occurring in peroxisomes and act by decomposing H_2O_2 into H_2O and O_2.

Nonenzymatic antioxidant systems are mainly formed by compounds of low molecular weight showing reducing functions or the ability to trap free radicals. In the first category, glutathione in its reduced form is considered the universal detoxificant (Vasseur and Leguille 2004). This triptide is an important antioxidant in eukaryote and prokaryote species. It acts as an electron donor directly able to inactivate several types of ROS. It also constitutes a substrate for enzymatic activity of GPx. Low levels of cellular GSH usually make the cells more sensitive to pro-oxidant compounds. The liposoluble vitamins E and A occurring in the cell membrane are able to capture some ROS as the superoxide anion or the hydroxyl radical right from their formation and then avoid the effects of oxidative stress.

Under stress conditions, the activity of antioxidant defense systems could be induced or inhibited. Usually, induction is interpreted as an adaptation of organisms faced by environmental disturbances, whereas inhibition reflects the toxic effect of pollutants and indicates cell damage (Cossu et al. 2000; Vasseur and Cossu-Leguille 2003). The measurement of antioxidant enzymes could give an indication of the organism's antioxidant status and could be used as a biomarker of oxidative stress. More generally, the assessment of the components of the antioxidant defense systems occurring among animals in different tissues, represents a nonspecific biomarker of the adverse effects of xenobiotics (Valavanidis et al. 2006). In the past decade, this assessment has been used more widely given the general ability of tissues to eliminate different forms of ROS as measured by the total oxyradical scavenging capacity (TOSC) method developed by Regoli et al. (2002a). This method presents advantages that provide to the organism or tissue in an integrated approach:

- A general view of the antioxidant status that could only be obtained with difficulty by the individual measurement of one or several components of the antioxidant systems;
- An antioxidant response against a specific kind of ROS (Monserrat et al. 2007).

The systems of antioxidant defense show seasonal variations in relation to temperature, reproductive cycle, and food availability (Manduzio et al. 2005) in different mollusk and fish species (Regoli et al. 2002b; Leiniö and Lehtonen 2005; Bocchetti and Regoli 2006; Ansaldo et al. 2007). Usually, the maximum antioxidant activities are recorded in spring. They decrease during summer and reach minimum values in winter. The variations of antioxidant systems are conversely proportional to lipid peroxidation, explaining the increased sensitivity of organisms during winter (Niyogia et al. 2001).

Over the two past decades, the literature on the use of antioxidant system response as a defense biomarker has been important (Regoli et al. 2011). In this framework, numerous invertebrate and vertebrate, marine, and freshwater species have been used as sentinels to evaluate the effects of several organic and mineral xenobiotics both under experimental and natural conditions. Today, these biochemical responses are associated with those at other levels of biological organization in species belonging to different trophic levels in a multibiomarker approach required to obtain an integrated evaluation of contaminant impact (Beliaeff and Burgeot 2002; Orbea et al. 2002; Roberts and Oris 2004; Aït Alla et al. 2006; Damiens et al. 2007).

2.3.7 Heat Shock Proteins

Heat shock proteins (Hsps) are ubiquitous proteins, widely conserved throughout the evolution of eukaryotes. They are named according to their apparent molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Schlesinger et al. 1982; Atkinson and Walden 1985; Moromoto et al. 1990), in particular HSP 40, 60, 70, and 90. The Hsp of lower molecular weight (8 kDa) is called ubiquitine. Cellular response to stress was reported for the first time by Ritossa (1962), who observed Hsp induction in the case of a very significant temperature rise, hence their name. Hsps are now called stress proteins because they are overexpressed in response to a certain number of physical and chemical factors including anoxia (Spector et al. 1986), salinity stress (Ramagopal 1987), metals (Hammond et al. 1982; Caltabiano et al. 1986), xenobiotics (Sanders 1990), and oxidative stress in general (Freeman et al. 1999).

Some Hsps are constitutive; for example, Hsp 60 and 70 are involved in the homeostasis of proteins under normal conditions while playing a protective and repairing role in the event of environmental stresses (Rothman 1989; Welch 1990). Stress proteins have a capacity to repair proteins harmed by stress or to eliminate them when they cannot be repaired any further. They work as molecular "chaperones," accompanying, monitoring, and protecting other proteins (Frydman 2001; Hartl and Hayer-Hartl 2002). They can act in the posttranslational spatial configuration of proteins and intervene in the transfer of proteins to the mitochondria, and in the induction and control of apoptosis (Craig et al. 1994; Creagh et al. 2000). Stress proteins and the genes that code for them have been sequenced in many organisms. Because of their sensitivity to environmental pollutants such as metals, several researchers quantified Hsp 60 and 70 in the bivalve sentinel species *M. edulis* (Sanders et al. 1991, 1994; Brown et al. 1995; Werner and Hinton 1999). Hsp levels reflect the physiological state of the animal.

Another group of proteins, that of glucose-regulated proteins (GPRs), has been discovered (Welch 1990; Hightower 1993). GPRs have very strong analogies with Hsps.

2.4 Damage Biomarkers

2.4.1 AChE Activity

The inhibition of cholinesterase activity can be regarded as one of the first biomarkers proposed in environmental monitoring studies, since its development in human medicine as an index of exposure to neurotoxins, in particular organophosphates from war gases, goes back several decades. For many authors, the measurement of AChE activity is the best marker of contamination by organophosphorous pesticides and carbamates (Holland et al. 1967; Coppage and Braidech 1976; Galgani and Bocquené 1989; Day and Scott 1990). Cholinesterases are enzymes that catalyze the hydrolysis of esters of choline more quickly than other esters. In vertebrates, two cholinesterases have been identified: AChE (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8, BuChE). AChE is inhibited by excess of substrate but BuChE is not. In spite of the limited number of genes apparently involved, ChEs present a large variety of molecular forms including globular (monomer, dimer, tetramer) and asymmetric forms (from 4 to 12 subunits with a collagen tail). At least eight forms of AChEs are found with a different oligomeric organization, solubility, and

mode of membrane anchorage in vertebrates (Mora et al. 1999). Some studies suggest that a polymorphism of ChEs may exist for mollusks. Indeed, two distinct ChEs differentiated by their solubility and their sensitivity toward organophosphates have been found in the oyster *C. gigas* (Bocquené et al. 1997). In some biomonitoring studies, it is not clear whether only AChEs or also pseudocholinesterases are able to hydrolyze the substrate (acetylthiocholine) used; thus, authors should choose to use the nonspecific term of cholinesterases when presenting biological monitoring results.

Measurements carried out on dab (the flatfish *Limanda limanda*) along a 360-km transect in the North Sea (Galgani et al. 1992) showed important inhibitions of various types of cholinesterases. This effect, mainly observed in animals coming from near the coast, is due to compounds carried from the estuaries of the Elba and Weser rivers. The identification of the inhibiting compounds of ChEs nevertheless remains delicate, and it is not possible to definitely conclude that organophosphorous and carbamates are the only chemicals responsible for the observed inhibition effects on ChEs in the various marine compartments. The chemical data on these products are scarce, and marine organisms are subjected permanently to the effects of complex mixtures of contaminants. Payne et al. (1996) wonder whether AChE activity is an old biomarker with a new future. Indeed, these authors show that an inhibition of AChE activity could be associated with an induction of EROD activity in the livers of trout (*Salmo trutta*) and flounders (*Pleuronectes americanus*) caught in an area contaminated with pulp mill effluents.

Contaminants other than pesticides can inhibit AChE activity. Leiniö and Lehtonen (2005) report inhibition of AChE by metals, detergents, and algal toxins. These authors conclude that the inhibition of AChE activity can be regarded as a marker of the physiological state of the animals. Moreover, Pfeifer et al. (2005) emphasize that AChE activity in mussels *Mytilus* sp. collected from Baltic Sea is negatively correlated with salinity. The abiotic parameters of the environment thus need to be taken into account as with other biomarkers when performing biological monitoring.

2.4.2 Vitellogenin

Biomarkers of endocrine disruption are used more and more since many studies have shown that the reproduction of fish is very sensitive to chemical pollutants. Among the chemical compounds reaching the aquatic environment, the first endocrine disruptor compounds (EDCs) were those acting as estrogens by their capacity to mimic the natural estrogen, estradiol, thus causing a feminizing action on organisms. The general term of EDCs now includes molecules of very varied structure and origin (PCBs, tributyltins, or natural phytoestrogens coming from the metabolism of soya or clover). The incidence of fish hermaphroditism close to wastewater treatment plants in the United Kingdom (Purdom et al. 1994) led to a study of the "estrogenicity" of the effluents of the treatment plants. Ethynylestradiol, a synthetic estrogen used as contraceptive, is involved in these effects (Purdom et al. 1994). Human natural estrogens (17β-estradiol, estriol, and estrone) and their conjugates, excreted in urine and feces, contribute to estrogenicity (Larsson et al. 1999). Another chemical molecule is nonylphenol, used as an intermediate in the industrial production of nonylphenol ethoxylates, a large group of nonionic surfactants widely used in plastics, latex paints, household and industrial detergents, and paper and textile industries (Lee 2002). However, according to Soto et al. (1995), EDCs mimic not only the sex steroid hormones estrogens but also androgens, by binding to hormone receptors or influencing cell signaling pathways; they block, prevent, and alter hormonal binding to hormone receptors or influence cell signaling pathways; they alter

production and breakdown of natural hormones and modify levels and function of hormone receptors.

When exposed to estrogens and "mimetic estrogens," the liver synthesizes vitellogenin (VTG), a lipoglycophosphoprotein (which is a precursor of yolk egg reserves) specific to females, regardless of the age of fish. VTGs are high-density (300–600 kDa, according to species) glycolipophosphoproteins having Ca and Zn ligands and are considered to have similar characteristics in vertebrates, such as fish (Nagler et al. 1987), and invertebrates, particularly mollusks (Blaise et al. 1999). The "estrogen mimics" exert a feminizing action, thus concerning *a priori* more male individuals with VTG induction, oocyte and oviduct presence in the testes, fecundity decrease, modification of the sex ratio, and reduction in the secondary sexual characters in the male (Tyler and Routledge 1998).

However, field measurements of effects on the reproduction of fish are far from clear; a full demonstration of any effect on fecundity and reproduction, size, or structure of fish populations indeed requires field investigations that are time consuming and spatially limited. The feasibility of the measurement of VTG and the interpretation of histological slides of gonads of male fish collected from French rivers was studied in the chub (*Leuciscus cephalus*) (Flammarion et al. 2000). First results have been followed by a large-scale field experiment with this species. Measurements have demonstrated moderate but significant VTG induction in chub collected downstream from large towns in France (Paris or Lyon). Iwanowicz et al. (2009) evaluated the reproductive status of smallmouth bass (*Micropterus dolomieu*) in the upper Potomac River and its tributaries. They noted the presence of immature female germ cells (oocytes) in the testes of some of the male fish. Further evidence of endocrine disruption occurred when the authors detected the presence of VTG in the blood of male fish. In addition to the effects on male fish, a substantial decrease in VTG in females also suggested endocrine disruption. At present, VTG is considered a biomarker of endocrine disruption in fish and some mollusks. In the freshwater mussel (*Elliptio complanata*), VTG concentrations in hemolymph and gonad increase after exposure to effluents from wastewater treatment plant (Gagné et al. 2001).

2.4.3 Lysosomal Membrane Stability

It is known that lysosomes play a significant role in the catabolism of cellular compounds, the intracellular transport of macromolecules, and the storage of metals (Viarengo et al. 1984) and of organic contaminants (Moore 1988). The lysosomal membrane is weakened in the liver or digestive gland of animals subjected to pollution. It is very difficult to evaluate the molecular changes affecting the permeability of the lysosomal membrane. Analyses of this permeability require extremely purified preparations of lysosomal membrane and their study at a molecular level (see Chapter 5). An easier way to evaluate this parameter is to examine whether its physiological function is changed or destroyed following an exposure to pollutants. Cytochemistry is the relevant tool that links descriptive morphology and biochemistry to observe such pathological modifications. This technique was used successfully to estimate the integrity of the lysosomal membrane by visualizing the hydrolytic enzymes inside the lysosome, and it proved to be a fast and sensitive research tool to evaluate the effects of different xenobiotics (Pellerin-Massicotte and Tremblay 2000). This unspecific response intervenes in all cellular types from fungi to vertebrates. Viarengo et al. (1995) showed that a short-term exposure to pollutants in micromolar amounts (ionic copper Cu^{2+}, dimethylbenzoanthracene, and Aroclor 1254) reduced the stability of the lysosomal membrane (LMS) of the digestive gland of mussels *M. galloprovincialis*. Broeg et

al. (2002) studied LMS in livers of the flounder (*Platichthys flesus*) from the North Sea; the lysosomal membrane was affected in fish from the Elba river between 1995 and 1999 but then recovered its integrity in 2000. On the other hand, fish from the Eider river or around Helgoland, which are located farther from pollution sources (DDT and PCB) than the Elba river, showed a decrease in the integrity of lysosomal membrane that has been constant between 1995 and 2000. The authors suggest that the fish populations not continuously exposed to anthropogenic stress have a lower potential or take longer time to recover a good physiological state.

2.4.4 Thiobarbituric Acid Reactive Substances

Deficiency of antioxidant defense systems to eliminate an excess of ROS could induce different types of cellular damage, of which the most widely studied is the peroxidation of lipids (Figure 2.2), able to induce structural and chemical alterations of cellular membranes (Livingstone et al. 1990; Winston and Di Giulio 1991; Vasseur and Cossu-Leguille 2003; Valavanidis et al. 2006). The process of lipid peroxidation involves a chain of reactions leading to the breakdown of polyunsaturated fatty acids that are relatively sensitive to oxidative reactions. Their degradation induces the formation of various compounds such as lipid alcoxyl radicals, ketones, alkanes, epoxides, and aldehydes. Among them, malondialdehyde (MDA) is both the most important and the most studied. Most of these compounds are toxic and mutagenic. The peroxidation of lipids could be initiated by hydroxyl radicals particularly in reactions catalyzed by transition metals (Viarengo et al. 1990; Valavanidis et al. 2006; Almeida et al. 2007).

The effects of lipid peroxidation can be assessed at the different steps of the lipid breakdown: at the initial phase (conjugated diene), intermediate phase (lipid hydroperoxides), or final phase [substances (TBARS) reactive with thiobarbituric acid (TBA) considered as MDA-like peroxides]. This test based on the use of these substances mainly reveals the formation of MDA by colorimetric or fluorimetric methods. Because TBA can react with compounds other than MDA, the results are usually expressed as TBARS concentrations (Knight et al. 1988; Pannuzio and Storey 1998; Durou et al. 2007).

The levels of MDA and TBARS have been used as markers of oxidative stress indicating the peroxidation of cellular membranes in numerous marine and freshwater invertebrate and vertebrate species. They can be influenced by different environmental parameters such as salinity and temperature in bivalves (Damiens et al. 2004) or in fish and can increase 20-fold in goldfish (*Carassius auratus*) exposed to a temperature elevation of 14°C (Lushchak and Bagnyukova 2006). In different populations of the same species, the levels of TBARS can show seasonal variations. In the estuarine polychaete (*Nereis diversicolor*), no variations were observed in the Seine estuary (Durou et al. 2007), but higher levels were recorded in January and October at different Moroccan sites (Aït Alla et al. 2006). In bivalves, no TBARS variations were observed in *Mytilus* sp. (Shaw et al. 2004; Bocchetti and Regoli 2006), whereas their concentrations were maximum in *Perna viridis* during spawning in May despite a strong activation of antioxidant systems (Wilhelm Filho et al. 2001). In marine bivalves, other environmental factors such as tidal cycles can influence lipid peroxidation, which increases during emersion (Durand et al. 2001; Almeida et al. 2005). On the contrary, these phases of immersion/emersion did not induce variations of TBARS in the gastropod *Littorina littorea*, whose antioxidant systems neutralize ROS formation during the aerial phase (Pannuzio and Storey 1998).

Moreover, numerous studies conducted during the past two decades in marine and freshwater media have shown that the levels of lipid peroxidation can be affected by

environmental pollutants belonging to different classes of a different nature (Cossu et al. 2000; Giguère et al. 2003; Roméo et al. 2003; Aït Alla et al. 2006; Damiens et al. 2007).

2.4.5 DNA Damage

As reported above, ROS continuously produced in aerobic organisms when not neutralized may cause deleterious cellular effects such as lipid peroxidation described in the previous paragraph, protein breakdown, or DNA base oxidation (Figure 2.2). The preservation of DNA molecule integrity is critical for all living organisms, and they possess efficient protective systems for their genetic material.

Between the first contact of a xenobiotic with the DNA molecule and a potential mutation, an event sequence is produced beginning with the direct or indirect formation of DNA adducts. The secondary modifications of DNA produced can be induced by an oxidative stress and correspond to a single- or double-strand breakdown, an increase of its repair level or base oxidation. When DNA disturbances become permanent, they can induce an alteration of cellular functions and uncontrolled proliferation leading to carcinogenesis. Finally, when the contaminant impact is observed during cell division, it can produce a mutation transmitted to future generations (Møller and Wallin 1998; Burcham 1999; Valavanidis et al. 2006; Almeida et al. 2007; Hwang and Kim 2007; Monserrat et al. 2007 and references quoted by these authors).

The detection and quantification of DNA damage allow its use as a biomarker of genotoxicity under acute or chronic conditions (Chapter 13). Usually, stress conditions induce cellular disturbances in organisms and an increase in DNA damage. Most of the recent published studies are focused on DNA damage induced by oxidative stress.

DNA oxidation generates different modified bases of which 8-oxo-7,8-dihydro-2′-deoxiguanosine (8-oxodGuo), produced by the reaction between oxygen and guanine, are the most measured in aquatic organisms by high-performance liquid chromatography. Other oxidized bases can be studied such as thymine glycol, 5-hydroxymethyluracil, formylamidopyrimidine, and 8-hydroxydeoxyadenine (Martinez et al. 2003; Hwang and Kim 2007).

The Comet test (SCG or single cell gel electrophoresis) is a quantitative technique, quick and visual, to measure DNA strand breakdown in eukaryote cells (Devaux et al. 1997; Burlinson et al. 2007). The method is based on migration during electrophoresis of damaged DNA from the nucleus, forming an impression of a comet, the head of which corresponds to the cell nucleus with intact DNA, whereas the tail is formed by the cut DNA strands. Recent modifications of this test specifically reveal the oxidized DNA bases (Hwang and Kim 2007).

Other DNA damages assessed as genotoxicity biomarkers involve the DNA adducts formed by the nucleotides on which the chemical mutagens are fixed (^{32}P postlabeling) and the mutation quantified at the chromosomal level by the micronucleus test (Monserrat et al. 2007).

More recent molecular biology techniques of DNA amplification (random amplified polymorphic DNA) or polymerase chain reaction have been used to assess the direct effects of xenobiotics on DNA, and also the genetic diversity of studied populations. Actually, these techniques still lack reproducibility and only with difficulty allow the separation of the two mechanisms (Atienzar and Jha 2006).

An increasing number of aquatic and terrestrial ecotoxicological studies include the measurement of different forms of DNA damage in order to evaluate the genotoxicity of physical and chemical environmental stress on plants or animals, whether vertebrates or