



# Biomarkers in Ecotoxicological Research Trails

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## Abstract

Biomarkers are demonstrated as novel strategies in ecotoxicological trails to implicate risk assessment and policy. Evaluation of the stressors in the organisms and/or wildlife passes with challenges for measuring risk factor. However, it is easy to assess the impact and adverse effects in target or non-target. Modern pattern and technologies in toxicological research were updated to provide multi-biomarkers categories. As stated, cytokines are employed as a novel biomarker especially that used for allergy screening, disease's diagnosis, and occupational exposure assessment. On the other hand, epigenetic marks on particular gene could serve as biomarkers for exposure to specific stressors or toxic outcomes. Biomarkers may rely impact of physical and chemical compounds or environmental changes on organisms. Most of biomarkers are enzymes, proteins and molecular molecules. So, sentinel animals models can serve as good indicators to state ecosystem health or stress's evidence during monitoring programs. Sum of biomarkers in correlation with stressors may provide early warning of potential risk to ecosystems.

**Keywords:** Biomarkers; Sentinel animals; Enzymes; Epigenetic; Proteomic

## Introduction

Biomarkers are defined as measurement of interaction between biological system and environmental agent, which may be chemical, physical, or biological [1]. Therefore, in vivo induction of biomarkers is a good environmental tool to assess the exposure and adverse effect of stressors on organisms [2-5]. The biological response of an organism to a stressor following uptake may induce changes at the cellular biochemical levels, resulting in alteration of the cell structure and function, tissues and behavior of the organism [6-8]. Biomonitoring can be conducted by sampling organisms living in the investigated areas (passive biomonitoring) or by exposure of organisms from either reference site or laboratorial culture to the investigated area (active biomonitoring) [9].

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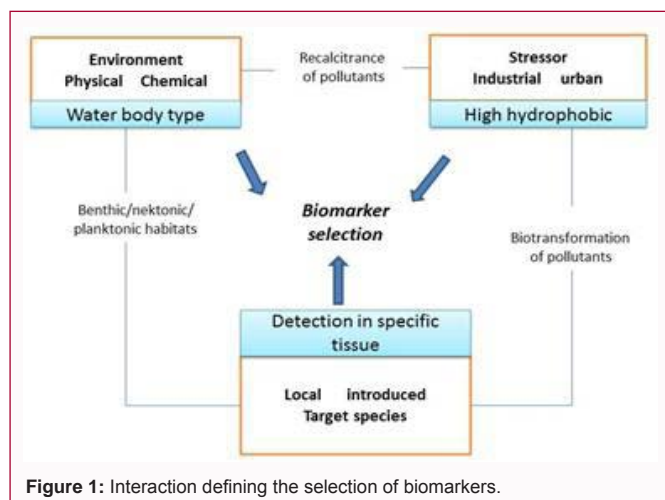
## Target Species

Sentinel animals models could involve mammalian or non-mammalian species, domestic animals, or wildlife. Results/findings of the studies may include mortality, developmental defects, reproductive effects, carcinogenicity, neurotoxicity, immunotoxicity, behavioral changes, and others [10]. These findings provide early warning of potential risk to ecosystems. Although it is unlikely that sentinel species data will be used as the sole determinative factor in assessing human health risk, but can serve as a nearly evidence for further studies. Furthermore, they can suggest potential causes and effects [11]. Bioindicators are generally understood to mean organisms or groups of species whose occurrence, behavior, habit, etc. are closely correlated with defined ecological factors. These factors can serve as direct indicators of the state of an ecosystem, stress on the ecosystem or changes in the ecosystem. There are some parameters usually employed for the analysis of indicators in monitoring programs:

- Change in the composition of species
- Presence or absence of key species
- Biomass (subdivided at least by ecosystem compartment)
- Biochemical stress indicators
- Pathology or parasitization of populations
- Analytical evaluation of bioaccumulation

## Reaction Indicators

This concept can provide information about the overall state of stress at a site, but not about



the substance which have been found there on the basis of chemical analysis. The selected species should have the following characteristics:

- Sensitivity to stress factors
- High density of individuals
- Easy to analyze/monitor
- Rapid success of generation
- Differentiation according to life forms or consumer type
- Confinement to certain geographical region

## Accumulation Indicators

The accumulation indicators may be plants, mussels or earthworm, but parts of higher organisms can also be used.

Accumulation indicators must meet the following conditions:

- Uptake of the foreign substance and concentration of the substance in relation to the ambient medium in due time
- Tolerance of the foreign substance to permit uptake
- No problems with the residue analysis
- Laboratory keeping and breeding are possible.

Thus, it becomes necessary to know the distribution, life cycle, and ecological traits of the native species present in ecosystems, since these conditions could increase the ecological relevance of biomarkers. Moreover, the selection of the target soil species is a key aspect in biomarker studies, and can determine its usefulness, but it can also induce mistakes when the final correlations are done. Most studies in aquatic environments use of motionless species e.g. mussels to avoid the preconception produced by the migrations and the changes from polluted sites to polluted-free sites [12-15]. For example, mosquito fish has a widespread occurrence in small streams, easy to culture in laboratory and has highly enzymatic activities. Thus, it is a potential sentinel to use as a regional bioindicator of environmental contamination [16].

Terrestrial snails are now considered as appropriate bioindicator in ecological risk assessment of chemical pollution. They have high reproduction rate and population density [17]. In addition, they are well adapted to warm climates and can survive long and rigid periods because they form a wall of dried mucus which reduces water loss

during dormancy.

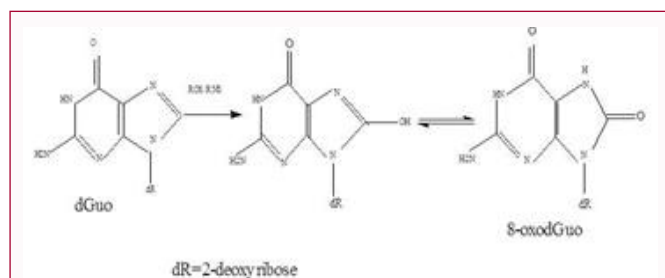
Earthworms represent the majority of total soil biomass and have favorable effects on soil structure and function. Pesticides and other contaminants in the natural habitat of earthworms can lead to an ecological imbalance [18-20]. Depending on the capacity to accumulate and concentrate large quantities of inorganic and organic pollutants, earthworm species are widely recognized as suitable organisms to monitor the effects of xenobiotic in contaminated soils [21,22]. Comparisons of biomarker responses and toxicant concentrations in soils lead to establish the sensitivity of the biomarker measurements and determine their value when used in conjunction with chemical data.

Microalgae have ecological significance attributing to their position at the base of the aquatic food webs. Those attribute the microalgae to be used as sentinel organisms in environmental studies in order to evaluate the toxicity of various chemicals or pollution discharges, and particularly inputs of metals [23-25]. Additionally, photosynthetic organisms such as algae are early and timely indicators of potential hazard in aquatic systems and should be seriously considered in any environmental assessment program [26]. Many studies depending on organisms had been reported as regionally important tools in environmental programs e.g. fish in Australia, Asia, America and Egypt [27-31], land snails [32,33,10], earthworm [34-39], macro algae [40-43], and microalgae [44-47]. Moreover, biochemical responses of the organisms exposed to Persistent Organic Pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and pesticides have been reported for the last two decades and documented by the international Organizations such as Economic Corporation and Development (OECD) and the United State Environmental Protection Agency (USEPA).

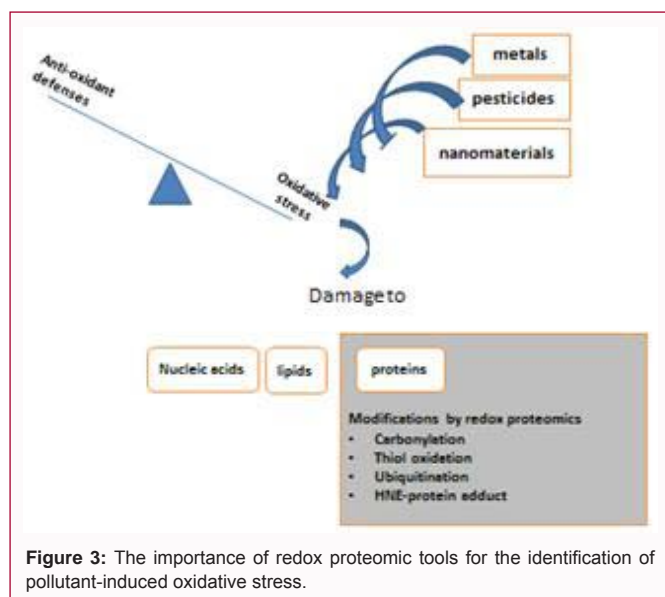
## Stressors

In ecosystem, the impact derived from different activities has changed depending on the distribution of molecules, ions, and compounds. Additionally, synthesis of new agro industrial and pharmacological compounds has increased the number of pollutants released into ecosystems [48]. Most of environmental pollutants may be grouped to; i) toxic organic compounds such as PAHs, Endocrine Disrupting Compounds (EDCs), Perfluorinated Compounds (PFCs), and alkyl phenols (APs), ii) metals, and iii) emerging pollutants such as pesticides and Pharmaceutical Active Compounds (PhACs). These groups have the ability to stay for a long-term in the ecosystems. This interaction allows chronic interactions of pollutants with human and wildlife and facilitates their accumulation through the skin and other tissues. For example, PAHs recalcitrance leads to the expression of cytochrome P4501A in mussels, even months after the first exposure episode [49]. In addition, they could be metabolized to active molecules-forming adducts. Others such as pesticides and PhACs are biotransformed by hepatic cells [50,48]. Metals are biopersistent in biological systems at levels greater than those in surrounding environment. This concept is recognized by formation of Metallothioniens (MTs) [51].

The study of biomarkers allows the identification of side effects-derived from pollutants in water and soil. On the other hand, each system is influenced by the weather, water flow and levels of contaminated stress as well as the interactions between pollutants and target species [14]. Seasonal variations induce changes in temperature and pH of the ecosystems. These changes allow microorganisms like



**Figure 2:** Oxidation of 2'-deoxyguanosine (dGuo) by ROS/RNS forming of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo).



**Figure 3:** The importance of redox proteomic tools for the identification of pollutant-induced oxidative stress.

cyanobacteria and algal blooms decrease the oxygen availability or the production of toxic substances that could induce the expression of proteins associated with oxidative damage, such as glutathione-S-transferase [52,53]. Climate changes could increase the bioavailability of PAHs, metals and increase the miRNA expression in fish exposed to some metals [54]. The physicochemical composition of the sediment and soil modulates the bioavailability of pollutants that finally enter into the trophic chain. Metal ions are retained depending on the clay proportion in soil and pH variations [55].

## Enzymes

Measurement of activity of target enzymes, hormones receptors, and neurotransmitters are commonly used as biomarkers for acute and short-term exposure to agrochemical as well as other toxic chemicals. The successful biomarker must be applicable under laboratory and field conditions [56]. Organophosphorus (OP) and Carbamate (CB) pesticides continue to be important classes of agrochemicals used in modern agriculture worldwide. So, they persist in the environment for a relatively short time, but they show a high acute toxicity that may represent a serious hazard for wildlife. They are specific inhibitors for Acetylcholinesterase (AChE, EC 3.1.1.7) in the nervous system. It is considered the most popular biomarker of OP and CB pesticide exposure in many vertebrate and invertebrate species [57,58]. Most of the studies concern ecotoxicological investigations involve the use of Cholinesterase (ChE) as a biomarker of exposure to these groups in aquatic organisms [30], marine organisms [59]. Despite, terrestrial snails are now considered as applicable bioindicator in ecological risk

assessment of xenobiotic pollution; few studies have investigated the lethal and sublethal effects of OP and CB pesticides in the terrestrial snails [59].

Since there is a close relationship between environmental stress and the rate of cellular reactive oxygen and nitrogen species (ROS/RNS) generation in the organism, ROS/RNS can be produced as by-products of cellular metabolism. If they are not immediately intercepted by antioxidant defenses, they could oxidize different cell components when the rate of production exceeds the rate of decomposition by antioxidant defenses and repair systems. Oxidative stress can be established leading to oxidation of key cell components (Figure 1) [60]. Exposure of organisms to pollutants can promote an increase in the rate of ROS/RNS production, thus the assessment of oxidative stress-related parameters in specific sentinel organisms could be included in environmental pollution monitoring studies to predict the impact of xenobiotic present in the environment. However, other environmental parameters not related to pollution, such as temperature, salinity, and others can cause important changes in biochemical system that have been reported as biomarker. Therefore, basic information about the influence of these factors on the biomarker to be used in different species is required to get accurate investigations [61].

Organisms have antioxidant defense mechanisms that prevent and intercept ROS/RNS, as well as repair mechanisms for oxidized components. Also, cells contain antioxidant enzymes that can intercept ROS/RNS, protecting molecular targets against oxidative injury. The three major antioxidant enzymes are the Superoxide Dismutase (SOD) which converts  $O_2^{\cdot -}$  to  $H_2O_2$ , Catalase (CAT) that catalyzes the decomposition of  $H_2O_2$  to molecular oxygen and water. Finally, glutathione peroxidase (GPx) which reduces both  $H_2O_2$  and lipid hydroperoxides associated to glutathione oxidation (GSH) [60]. Enzymes involved in the transport and elimination of reactive compounds carry other indirect antioxidant functions e.g. glutathione-S-transferase and the transport systems for the glutathione-S-conjugates. Non-enzymatic defenses include the fat-soluble vitamins,  $\alpha$ -tocopherol, and  $\beta$ -carotene, as well as some low molecular weight compounds like glutathione content (GSH).

On the other hand, cell membranes are potential targets of attack by ROS/RNS. The attack of membrane lipids by these species initiates an oxidation process known as lipid peroxidation (LPO). The formation of LPO in membranes disrupts the normal cellular metabolism, triggering adaptive responses and/or causing cell death [62]. Another oxidative damage breaks a number of different modified DNA bases. Recently, the evaluation of modified DNA bases, especially 8-oxodGuo levels has proved to be a good indicator of oxidative stress caused by xenobiotic exposure (Figure 2). The oxidized DNA induced in different experimental models by  $\cdot OH$  and  $\cdot O_2$  is considered as a fingerprint to oxyradicals attack to DNA [63]. The use of oxidative stress biomarkers is of potential interest for the assessment of the pollutants impact or seasonal variations in animals under field conditions [64,65]. Moreover, the interaction between xenobiotic and the components of the antioxidant defense systems play an important role in ecotoxicological response of an organism to its environment [32].

Biotransformation enzymes participate in the solubilization and removal of xenobiotic to avoid its bioaccumulation and entering the trophic chain. Monooxygenases of the cytochrome P450A are a complex (CYP1A1). Its role is the metabolism of xenobiotic, catalyzing

**Table 1:** Biomarkers used in environmental risk assessment studies.

Group	Name	Abberv.	Biological processes	References
Biotransformation Enzymes	Eukaryotic	CYP1A	Exposure/transformation of aromatic and planar organochlorine compounds	[14,68,69]
	Cytochrome P450			
	Ethoxyresorufin-o-deethylase	EROD	Exposure/biotransformation of xenobiotics such as PAHs, PCBs and PCDDs	[53]
	Glutathione-S-transferase	GST		
	Glutathione	GSH		
Oxidative and cellular stress proteins	Superoxide dismutase	SOD	Over expression in some tissues such as gills, but mainly in liver after the detoxication process	[70]
	Catalase	CAT		
	Glutathione	GPx		
	Peroxidase			
	Glutathione	GR		
	Reductase	POD		
	Peroxidase			
	Lactate dehydrogenase	LDH	Cellular lysis and tissue damage	[71]
	Heat shock protein 70	HSP70	Produced in response to chemical or physical stressors	
Metal-binding cysteine-rich proteins	Metallothioneins	MTs	Compensatory mechanism during exposure to heavy metals (Cd, Fe, Hg, Zn, As)	[72,73]
Endocrine system proteins	Vitellogenin	VTG	Alteration or disruption of hormonal axis by mimicry	[74,14]
	Vitelline envelope proteins	VEPs		
	Cytochrome P450	CYP19A		
	Aromatase			
	Acyl Coenzyme A oxidase	ACOX1	Produced after exposure to oil hydrocarbons	[75]
Programmed cell death proteins	Direct 1AP-binding mitochondrial protein	DIABLO (SMAC)	Found in liver after exposure to PCBs	[76]
	Caspases	CASP2-10	Initiators of programmed cell death when the stressor overcome rescue mechanismsd in the cell	[76]
DNA integrity markers	Micronuclei		It can overcome 8.8 times its expression after oil spills in mussels	[77]
	DNA adducts		Exposure of DNA to intercalating aromatic planar compounds	[78,14]
	DNA fragmentation			

the oxidative biotransformation, reduction and hydrolysis of the substrates. This concept explains that, these nonpolar compounds are converted into more water-soluble molecules that may be excreted by the organism. In aquatic ecosystem, CYP1A is widely monitored in fish and mollusks which described a strong association of the expression and the presence of aromatic hydrocarbons [66]. In mussels exposed to oil spills, CYP1A levels begin to increase on month after exposure and remain detectable after six months [49].

## Protein

Many environmental pollutants can alter cell redox balance and induce oxidative proteins. They induce the generation of ROS/RNS and cause serious toxicity independent of quick interaction with biomolecules. Oxidation of proteins can be regarded either as negative consequence of a stress conditions (especially, irreversible protein oxidative modifications such as carbonylation, oxidation of cysteine to sulphonic acids, oxidation of tryptophan) or specific signal for the cell to respect to such stress (Figure 3). Despite, the environmental and occupational settings may generate free radicals, an accurate demonstrations of their effects at molecular level has started to be addressed [67]. Several research groups have pioneered a proteomic approach for ecotoxicology. This field is rapidly growing and gaining interest within the scientific community.

Carbonylated proteins are considered the best characterized

category of oxidized proteins. Protein identification can be followed by gel-based or gel-free methods. The main used method depends on derivatization with specific reagents such as 2,4-Dinitrophenylhydrazine (DNPH) or biotin hydrazide. The products may be subjected to reversed phase chromatography coupled with mass spectrometry (RPC-MS/MS) or ion exchange and IEC/RPC-MS/MS. Similarly, avidin can be used to detect biotinylated proteins by affinity chromatography or ELISA technique (Table 1).

Heavy metal-associated proteins are high molecular weight proteins rich in cysteine and sulphhydryl groups, which interact with metal ions and induce the transformation in the system. This kind of protein expression is named Metallothions (MT). The gene coding of protein was observed in organisms exposed to heavy metals. For example, overexpression of metalloproteins mRNA has been detected in fish and shellfish exposed to high concentrations of zinc (Zn), aluminum (Al), copper (Cu), lead (Pb), and cadmium (Cd) [70].

The new protein Chip technology allows fast and easy biomarker screening, particularly by processing large sets of individual samples. Additionally, SELDI-TOF-MS can be applied to investigate the effects of environmental contaminants and to establish new sensitive biomarkers for a variety of biological samples and contaminants. However, few toxicogenomic studies exist using the protein Chip technology aimed at identification pollutant-specific fingerprints. Differential profiling of human serum proteins has been performed



following exposure to heavy metals such as tin (As) and Pb [79-82] or to benzene [83]. Additionally, the coupling of protein Chips with MS/MS for the identification of proteins will provide researchers with a newer and powerful tool for biomarkers discovery. For these reasons, SELDI-TOD technology could represent analytical tools important to be promising.

## Molecular Biomarkers

### Cytokines

Inflammation is an adaptive response involving soluble mediators and specialized immune cells that is triggered in response to infections, toxicants or other forms of injury (Medzhitov, 2008). For example, Engineering Nanomaterials (ENMs) may act as inducers and stimulate the production of inflammatory mediators by innate immune cells. The variability in inflammatory responses to different ENMs may give prediction patterns of cytotoxicity coupled with pro-inflammatory cytokine responses. Recently, occupational exposure to toxicants can cause both pulmonary and airway-inflammation. Exposure to organic compounds e.g. PAHs and carbon black particles together resulted in changes in secretion of cytokine; IL-8 (Goulaswe et al). Proteomics and toxicoproteomics can increase the speed and sensitivity of toxicological screening by identification of protein markers related to inducers. Proteomics techniques may help to identify new molecular targets for toxicants or provide novel identifications into mechanisms of action. Such patterns or fingerprints could be used. Regarding this reason, Luminex technology can be used in proteomic examination for its potential in discovery of new biomarkers and toxicity signatures, in mapping serum, plasma, and other biofluid proteomes, and transcriptomic studies. It expands the range of analytes measured in a single sample to as many as 100 using microspheres in solution phase as a solid support for trapping the antibodies. The Luminex Lab MAPTM system is a powerful tool to use in proteomic biomarker profiles for toxicity prediction or occupational exposure assessment.

### Epigenetics

The field of epigenetics addresses how genes and the environment interact to form the basis of heredity and comes up with some surprising findings. The two types of epigenetic marks that have received the most attention are DNA methylation and histone modifications. They are essential to establish patterns of gene expression in the early embryo and often persist throughout life [84]. DNA (deoxyribonucleic acid) methylation and histone modification have been identified as important factors in epigenetic depending on regulation DNA methylation involves the addition of a methyl group to the 5' carbon of cytosine in a CpG sequence (cytosine-phosphate-guanine). Histone tails can also be conveniently modified by a number of different processes, e.g. methylation, acetylation, phosphorylation. Finally, microRNAs (miRNAs), a large family of non-coding RNAs (ncRNAs) that are evolutionarily conserved, endogenous and 21-23 nucleotides in length, need to be taken into accounts miRNAs regulate gene expression by targeting messenger RNAs (mRNAs) by binding to complementary regions of targeted transcripts to repress their translation or trigger mRNA degradation. They are encoded by the genome, and more than 1000 human miRNAs have been modified so far.

Certain stressors such as radiation and some chemicals can alter patterns of gene expression by mutation of DNA directly, but these events are relatively rare. In fact, chemicals and other environmental

factors have not disrupted gene expression by any mechanisms other than mutation. However, epigenetics may be addressed only in cases, where changes to gene expression are heritable (either mitotically or meiotically). This characteristic suggests that transient exposures can have important consequences at later life stages and also potentially affect the health of offspring. The epigenome acts as an interface between the changing environment and the genome, which is still very stable and resistant to environmental influences. However, typical environmental alterations to gene expression will generally decrease once a stressor is removed; epigenetics effects can persist as cells divide and even into successive generations.

In fact, chemicals can cause epigenetic effects. Different classes of environmental contaminants including metals, EDCs, organohalogenes, and solvents have been linked to epigenetic effects. In some cases, stressor-induced epigenetic modifications have been associated with negative health outcomes. Also, epigenetic effects occur at a range of contaminant levels, including very low concentrations of chemicals. The field of ecotoxicology has a lot to offer in this regard. Several model organisms can be studied in the laboratory under controlled conditions, and also in the fields under real-life exposure to multiple stressors. Most research on epigenetic effects of environmental chemicals have been done on rat, mouse and cell line, but epigenetic effects of stressors are also observed in ecologically relevant organisms. Such epigenetic changes (altered DNA modification and covalent histone modifications) may take place at the earliest stages of carcinogenesis and the identification of related markers which hold great promise for both risk assessments of chemical safety and biomedical research (Park, 2011). OECD recently stated proposal to develop work plan for assessing identification of biomarkers for genotoxic and/or non-genotoxic effects including epigenetics in systems of toxicology. These outputs may be provide and identify cancer prediction markers which could be useful for categories of chemicals in addition to genotoxicity test methods currently implemented.

Scientific evidence stated that, human domestic animals, wildlife and fish have been imposed adverse health effects mediated chemicals, including pesticides. These pesticides are referred to be as ED [85]. It is surprising that pesticides could have an ED potential, a compromise concern their mode of ED action and their acceptable risk assessment protocols, guidelines, methodologies and endpoints remains indefinable to a great extent [86]. Many problems have been detected in domestic or wildlife species exposure to some organochlorine compounds, PCBs, dioxins and some naturally occurring blow estrogens. Environmentally-polluting chemicals such as pesticides, can induce changes in DNA methylation in adults, influence their susceptibility to different pathologies and propagate diseases decades later in their offspring that were only exposed during prenatal and early life [87].

The concepts behind epigenetic have stirred up ideas in ecotoxicology from the basic understanding of mechanisms of action, to risk assessment and policy. The most considerable concept of epigenetics related to ecotoxicology is that, epigenetic mechanisms can create a temporal disconnect between exposure and effects. Exposure to chemicals early in development can leave epigenetic marks that result in disease later in life. Additionally, chemical-induced epigenetic modifications invited in one generation may be accepted to future generations in the absence of the initial stressor. Similarly, chemicals with an epigenetic modification can leave a lasting mark

that alters cell function, even when the chemical is no longer present [88-90]. The concept of a lasting epigenetic signal is highly relevant to biomonitoring and ecological risk assessment. Biomonitoring is used to assess exposure levels by direct measurement in target tissues. Along with information about the chemical's hazard, these values can support estimates of risk. Unfortunately, specific-hazard data are lack for many chemicals; the epigenetic effects are practically unknown. To assess risk in the absence of specific hazards, scientists often employ correlation approaches, linking chemical exposures with predictors of population health or biomarker responses. Epigenetic marks on particular gene could serve as biomarkers for exposure to specific stressors or toxic outcomes. In addition, epigenetic mechanisms may provide explanations for unresolved observations in the field of ecotoxicology.

## References

- WHO/IPCS. Environmental Health Criteria 155, Biomarkers and risk assessment: concepts and principles. IPCS, World Health Organization, Geneva. 1993.
- Dembêlê K, Haubruge E, Gaspar Ch. Recovery of acetylcholinesterase activity in the common Carp (*Cyprinus carpio* L.) after inhibition by organophosphate and carbamate compounds. *Bull Environ Contam Toxicol*. 1999;62(6):731-42.
- Ozmen M, Sener S, Mete A, Kucukbay H. In vitro and in vivo acetylcholinesterase inhibition effect of new classes of organophosphorus compounds. *Environ Toxicol Chem*. 1999;18(2):241-6.
- Sturn A, Wogram J, Segner H, Liess M. Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickles back (*Gasterosteus aculeatus*): application on biomonitoring. *Environ Toxicol Chem*. 2000;19(6):1607-17.
- Varô I, Navarro JC, Amat F, Guilhermino L. Characterization of cholinesterase and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemiasalina* and *Artemiapharthenogenetica*. *Chemosphere*. 2001;48(6):563-9.
- Lam PK, Gray JS. The use of biomarkers in environmental monitoring programs. *Mar Pollut Bull*. 2003;46(2):182-6.
- Eggen RI, Behra R, Burkhardt-Holm P, Escher BI, Schweigert N. Challenges in ecotoxicology. *Environ Sci Technol*. 2004;38(3):58A-64A.
- Moore MN, Depledge MH, Readman JW, Paul Leonard DR. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *Mutat Res*. 2004;552(1-2):247-68.
- Frangze O. Complex bioindication and environmental stress assessment. *Ecol Indic*. 2006;6(1):114-36.
- Abdel-Halim KY, Abo El-Saad AM, Talha MM, Hussein AA, Bakry NM. Oxidative stress on land snail *Helix aspersa* as a sentinel organism for ecotoxicological effects of urban pollution with heavy metals. *Chemosphere*. 2013;93(6):1131-8.
- Van der-Shalie WH, Gardner, Bantle JA, De Rosa CT, Finch RA, Reif JS, et al. Animals as sentinels of human health hazards of environmental chemicals. *Environ. Health Perspect*. 1999;107(4):309-15.
- Auslander M, Yudkovski Y, Chalifa-Caspi V, Herut B, Ophir R, Reinhardt R, et al. Pollution-affected fish hepatic transcriptome and its expression patterns on exposure to cadmium. *Mar Biotechnol*. 2008;10(3):250-61.
- Regoli F, Giuliani ME, Benedetti M, Arukwe A. Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquat Toxicol*. 2011;105(3-4 Suppl):56-66.
- Costa J, Ries-Henriques MA, Castro LF, Ferreira M. ABC transporters, CYP1A and GSTa gene transcription patterns in developing stages of the Nile tilapia (*Oreochromis niloticus*). *Gene*. 2012;506(2):317-24.
- Dallas LJ, Jha AN. Applications of biological tools of biomarkers in aquatic biota: A case study of the Tamar estuary, South West England. *Mar Pollut Bull*. 2015;95(2):618-33.
- Rendon-von Osten J, Ortiz-Arana A, Guilhermino L, Soares AMV. In vivo evaluation of three biomarkers in the mosquitofish (*Gambusia yucatana*) exposed to pesticides. *Chemosphere*. 2005;58(5):627-36.
- Mead AR. In: Fretter V, Peak J. (Eds.) *Economic Malacology with particular reference to Achatina fulicula*. Pulmonates. Academic Press New York, USA. 1979.
- Luo Y, Zang Y, Zhong Y, Kong ZM. Toxicological study of two novel pesticides on earthworm *Eisenia fetida*. *Chemosphere*. 1999;39(13):2347-56.
- Rao JV, Pavan YS, Madhavendra SS. Toxic effects of chlorpyrifos on morphology and acetylcholinesterase activity in the earthworm, *Eisenia fetida*. *Ecotoxicol Environ Saf*. 2003;54(3):296-301.
- Weber GBC. The role of earthworms as biological indicators of soil contamination. *Bull USAMV-CN*. 2007;64:1-2.
- Reddy NC, Rao JV. Biological response of earthworm, *Eisenia fetida* (Savigny) to an organophosphorus pesticide, profenofos. *Ecotoxicol Environ Saf*. 2008;71(2):574-82.
- Peijnenburg WCJ, Vijver MG. Earthworms and their use in ecotoxicological modeling. In: Devillers, J. (Ed.) *Ecotoxicology Model*. Springer, New York, 2009;177-204.
- Wei D, Kisuno A, Camella T, Urano K. A new method for evaluating biological safety of environmental water with algae, daphnia and fish toxicity ranks. *Sci Total Environ*. 2006;371(1-3):383-90.
- Labra M, Bemasconi M, Grassi F, et al. Toxic and genotoxic effects of potassium dichromate in *Pseudokirchneriella subcapitata* detected by microscopy and AFLP marker analysis. *Aquatic Bot*. 2007;86(3):229-35.
- Liebig M, Schmidt G, Bontje D, et al. Direct and indirect effects of pollutants on algae and algivorous ciliates in an aquatic indoor microcosm. *Aquatic Toxicol*. 2008;88(2):102-10.
- Kowalewska G. Phytoplankton the main factor responsible for transport of polynuclear aromatic hydrocarbons from water to sediments in the Southern Baltic ecosystem (extended abstract). *J Mar Sci*. 1999;565:219-22.
- Edwards JW, Edyvane KS, Boxal VA, Hamann M, Soole KI. Metal levels in section and marine fish flesh near industrial and metropolitan centers in south Australia. *Mar Pollut Bull*. 2001;42(5):389-96.
- Ueno D, Watanabe M, Subramanian A, Tanaka H, Fillmann G, Lam PK, et al. Global pollution monitoring of polychlorinated dibenzo-P-dioxins (PCDDs) furans (PCDFs) and coplanar polychlorinated biphenyls (coplanar PCBs) using skipjack tuna as bioindicator. *Environ Pollut*. 2005;136(2):303-13.
- Carrasco-Letelier L, Eguren G, Mello FT, Groves P. Preliminary field study of hepatic porphyrin profiles of (*Astynaz fasciatus*)(Teleostei, Characiformes) to define anthropogenic pollution. *Chem*. 2006;62:1245-52.
- Bakry NM, Osman KA, El-Aswad AF, Abdel-Halim Kh Y, Abou-Donia MB. Biomonitoring of pesticide contamination from the pesticide industry. *J Egypt Soc Toxicol*. 2001;24:107-11.
- Abdel-Halim KY, Salama AK, El-Khateeb EN, Bakry NM. Organophosphorus pollutants (OPP) in aquatic environment at Damietta governorate, Egypt: Implication for monitoring and biomarker responses. *Chemosphere*. 2006;63(9):1491-8.
- Regoli F, Gorbi S, Fattorini D, Sara Tedesco, Alessandra Notti, Nicola Machella, et al. Use of the land snail *Helix aspersa* as sentinel organism for monitoring ecotoxicological effects of urban pollution: an integrated approach. *Environ. Health Perspect*. 2006;114(1):63-9.

33. Radwan MA, El-Gendy KS, Gad AF. Biomarkers of oxidative stress in the land snail *Theba pisana* for assessing ecotoxicological effects of urban metal pollution. *Chemosphere*. 2010;79(1):40-6.
34. Gikutovic MA. Sperm counter in earthworms (*Lumbricusterrestnis*) as a biomarker for environmental toxicology: effects of cadmium and chlordane. *Environ Pollut*. 1993;81(2):123-5.
35. Erstfeld KM, Snaw-Ashbrook J. Effect of chronic low-level PAH contamination on soil invertebrate communities. *Chemosphere*. 1999;39(12):2117-39.
36. Hankard PK, Svendsen C, Wright J. Biological assessment of contaminated land using earthworm biomarkers in support of chemical analysis. *Sci Total Environ*. 2004;330(1-3):9-20.
37. Ecom IC, Rasti G, Veber AW, Vasseur P. Ecotoxicity of polycyclic aromatic hydrocarbons in contaminated soil. *Ecotoxicol Environ Saf*. 2007;67(2):190-205.
38. Kilic GA. Histopathological and biochemical alterations of the earthworm (*Lumbricusterrestnis*) as biomarker of soil pollution along Porsuk River Basin (Turkey). *Chemosphere*. 2011;83(8):1175-80.
39. Abdel-Halim KY. Earthworm (*Alloobophora caliginosa*) as a bioindicator of urban pollution with pesticides and potential toxic metals. *Sci Afric J Scient Issues Res Essays*. 2014;2(7):292-301.
40. Fytinaos K, Evgenidou E, Zachariadis G. Use of macro algae as biological indicators of heavy metals pollution in Thermaikos Gulf, Greece. *Bull Environ Contam Toxicol*. 1999;62(5):630-7.
41. Sanchez-Rodriguez I, Huerta-Diaz MA, Choumiline E, Holguin-Quinones O, Zertuche-Goneza JA. Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: implications for the geochemical control of metals in algal tissue. *Environ Pollut*. 2001;114(2):145-60.
42. Conti ME, Cecchetti G. A biomonitoring study: trace metals in algae and mollusks from Tyrrhenian coastal areas. *Environ Res*. 2003;93(1):99-112.
43. Abdel-Halim KY. Biomarkers of freshwater algae *Lemna minor* as a model for urban pollution with pesticides and heavy metals. *Alex Sci Exch J*. 2014;35(2):95-106.
44. Siripornadulsil S, Traina S, Verma DPS, Sayre RT. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell*. 2002;14(11):2827-47.
45. Nishikawa K, Yamakoshi Y, Uemura I, Tominaga N. Ultrastructural changes in (*Chlamydomonas acidophila*) (*Chlorophyta*) induced by heavy metals and polyphosphate metabolism. *FEMS Microbiol Ecol*. 2003;44(2):253-9.
46. Pinto E, Sigaud-Kutner TCS, Leitao MAS, Okamoto OK, Morse D, Colepiccolo P. Heavy metal induced oxidative stress in algae. *J Phycol*. 2003;39(6):1008-18.
47. Tripathi BN, Metha SK, Amar A, Gaur JP. Oxidative stress in (*Scenedesmus*) during short-and long-term exposure to Cu<sup>2+</sup> and Zn<sup>2+</sup>. *Chemosphere*. 2006;62(4):538-44.
48. De Castro-Catal N, Muñoz I, Armendriz L, Campos B, Barceló D, López-Doval J, et al. Invertebrate community responses to emerging water pollutants in Iberian river basins. *Sci Total Environ*. 2015;503-504:142-50.
49. Sureda A, Box A, Tejada S, Blanco A, Caixach J, Deudero S. Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). *Aquat Toxicol*. 2011;101(3-4):540-9.
50. Thompson E, Burwinkel KE, Chava AK, Notch EG, Mayer GD. Activity of phase I and phase II enzymes of the benzo[a]pyrene transformation pathway in Zebrafish (*Danio rerio*) following waterborne exposure to arsenite. *Comp Biochem Physiol C Toxicol Pharmacol*. 2010;152(3):371-8.
51. Le TTY, Zimmermann S, Sures B. How does the metallothionein induction in bivalves meet the criteria for biomarkers of metal exposure? *Environ Pollut*. 2016;212:257-68.
52. Falfushynska H, Gnatyshyna LL, Farkas A, Vehovszky A, Gyori J, Stoliar OB. Vulnerability of biomarkers in the indigenous mollusk *Anodontacyena* to spontaneous pollution in a transition country. *Chemosphere*. 2010;81(10):1342-51.
53. Cheng WX, Liang XF, Shen D, Zhou Q, et al. Seasonal variation of gut Cyanophyta contents and liver GST expression of nud carp (*Cirrlunanolitorea*) and Nile tilapia (*Oreochromis niloticus*) in the tropical Xiangang Reservoir (Huizhou, China). *Chin Sci Bull*. 2012;57:615-22.
54. Van Cleef-Toedt K, Crivello J, Kaplan LAE. Killifish metallothionein messenger RNA expression following temperature perturbation and cadmium exposure. *Cell Stress Chaperones*. 2001;6(4):351-9.
55. Boshalf M, Jordaens K, Baguet S, Bervoet L. Trace metal transfer in a soil plant-snail microcosm field experiment and biomarker responses in snails. *Ecol Indic*. 2014;48:636-48.
56. Griffith J, Duncan RC, Holka BS. *Arch Environ Health*. 1989;44:375-81.
57. Fulton MH, Key PB. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ Toxicol Chem*. 2001;20(1):37-45.
58. Van der Oost R, Beyerm J, Vermeulen NPE. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol*. 2003;13(2):57-149.
59. Brown TM, Bryson PK. Selective inhibitors of methyl parathion resistant acetylcholinesterase from *Heliothis virescens*. *Pestic Biochem Physiol*. 1992;44(2):155-64.
60. Sies H. Strategies of antioxidant defense. *Europ J Biochem*. 1993;215(2):213-9.
61. Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar Pollut Bull*. 2001;42(8):656-66.
62. Girotti AW. Lipid hydroperoxide generation, turnover, and effect or action in biological systems. *J Lipid Res*. 1998;39(8):1529-42.
63. Halliwell B. Oxidative DNA damage: meaning and measurement. In: Halliwell, B., Auroma, O.I. (Eds.), *DNA and Free Radicals*. Ellis Horwood, New York, 1993;67-79.
64. Regoli F, Principato G. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis* exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquat Toxicol*. 1995;31(2):143-64.
65. Verlecar XN, Jena KB, Chaiung GBN. Seasonal variation of oxidative biomarkers in gills and digestive gland of green-lipped mussel, *Perna viridis* from Arabian Sea. *Estuar Coast Shelf Sci*. 2008;76(4):745-52.
66. Bonnineau C, Saque IG, Urrea G, Guasch H. Light history modulates antioxidant and photosynthetic responses of biofilms to both natural (light) and chemical (herbicides) stressors. *Ecotoxicol*. 2012;21(4):1208-24.
67. Lemos MF, Soares AM, Correia AC, Esteves AC. Proteins in ecotoxicology-how, why and why not? *Proteomics*. 2010;10(4):873-87.
68. Liu D, Pan L, Cai Y, Li Z, Miao J. Response of detoxification gene mRNA expression and selection of molecular biomarkers in the clam *Ruditapes philippinarum* exposed to benzo[a]pyrene. *Environ Pollut*. 2014;189:1-8.
69. Liu H, He J, Zhao R, Chi C, Bao Y. A novel biomarker for marine environmental pollution of pi-class glutathione-S-transferase from *Mytilus coruscus*. *Ecotoxicol Environ Saf*. 2015;118:47-54.
70. Giarratano E, Gil MN, Malanga G. Biomarkers of environmental stress in gills of ribbed mussel *Aulacomya atra* (Nuevo Gulf, Northern Patagonia). *Ecotoxicol Environ Saf*. 2014;107:111-19.

71. Lehnert K, Mller S, Weirup L. Molecular biomarkers in grey seals (*Halichoerus grypus*) to evaluate pollutant exposure, health and immune status. *Mar Pollut Bull.* 2014;88(1-2):311-8.
72. Roesijadi G, Rezvankhah S, Perez-Matus A, Mittelberg A, Torruellas K, Van Veld PA. Dietary cadmium and benzo (a) pyrene increased intestinal metallothionein expression in the fish *Fundulus heteroclitus*. *Mar Environ Res.* 2009;67(1):25-30.
73. Ghedira J, Chicano-Glvez E, Fernndez-Cisnal R. Using environmental proteomics to assess pollutant responses of *Carcinus maenas* along the Tunisian coast. *Sci Total Environ.* 2016;541:109-18.
74. dosAnjos N, Schulze T, Brack W, Val AL, Schirmer K, Scholz S. Identification and evaluation of cyp1a transcript expression in fish as molecular biomarker for petroleum contamination in tropical freshwater ecosystems. *Aquatic Toxicol.* 2011;103(1-2):46-52.
75. Ruiz P, Ortiz-Zarragoitia M, Orbea A, Theron M, Le Floch S, Cajaraville MP. Responses of conventional and molecular biomarkers in turbot *Scophthalmus maximus* exposed to heavy fuel oil no. 6 and styrene. *Aquat Toxicol.* 2012;116-117:116-28.
76. Zacchino V, Minghetti M, Centoducati G, Leaver MJ. Diablo/SMAC: a novel biomarker of pollutant exposure in European flounder (*Platichthys flesus*). *Ecotoxicol Environ Saf.* 2012;79:176-83.
77. Barsiene J, Rybakovas A, Garnaqa G, Andreikenaite L. Environmental genotoxicity and cytotoxicity studies in mussels before and after an oil spill at the marine oil terminal in the Baltic Sea. *Environ Monit Assess.* 2012;184(4):2067-78.
78. Santos J, Meyer JN, Mandavilli BS, Van Houten B. Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. *Methods Mol Biol.* 2006;314:183-99.
79. Moore LE, Pfeiffer R, Warner M, Clark M, Skibola C, Steinmou C, et al. Identification of biomarkers of arsenic exposure and metabolism in urine using SELDI technology. *J Biochem Mol Toxicol.* 2005;19(3):176.
80. Zhai R, Su S, Lu X, Liao R, Ge X, He M, et al. Proteomic profiling in the sera of workers occupationally exposed to arsenic and lead: identification of potential biomarkers. *Biometals.* 2005;18(6):603-13.
81. Craddock RM, Huang JT, Jackson E. Increased alpha-defensins as a blood marker for schizophrenia susceptibility. *Mol Cell Proteomics.* 2008;7(7):1204-13.
82. Zhao L, Gao Y, Yang Y, Wei Y, Li Y, Feng H, et al. Serum proteomic profiling analysis of chronic arsenic exposure by using SELDI-TOF-MS technology. *Toxicol Lett.* 2010;195(2-3):155-60.
83. Vermeulen R, Lan Q, Zhang I, Gunn L, McCarthy D, Woodbury RL, et al. Decreased levels of CXC-chemokines in serum of benzene-exposed workers identified by array-based proteomics. *Proc Natl Acad Sci USA.* 2005;102(47):17041-6.
84. Head JA, Dolinoy DC, Basu N. Epigenetics for ecotoxicologists. *Environ Toxicol Chem.* 2012;31(2):221-7.
85. Mnif W, Hassine AIH, Bouaziz A, Bartegi A, Thomas O, Benoit Roig, et al. Effect of endocrine disruptor pesticides: a review. *Int J Environ Res Public Health.* 2011;8(6):2265-303.
86. McKinlay R. Endocrine disrupting pesticides-more precaution needed. *Risk assessment Pesticides News.* 2007.
87. Kankerkar RR, Bhatia Dey N, Csokam AB. Epigenetic across the human lifespan. *Front Cell Dev Biol.* 2014;2:49.
88. Carney JM, Starke-Reed PE, Oliver CN. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl phenylnitrone. *Proc Nat Acad Sci USA.* 1991;88:3633.
89. Davies KJA. Protein damage and degradation by oxygen radicals. *Int J Biol Chem.* 1987;262(20):9895-901.
90. Park S. Epigenetic marker as a component for systems toxicology useful for cancer prediction in addition to genotoxicity ECETOC Workshop Report No 3 Epigenetic and Chemical Safety 5-6 Dec 2011, Rome, Italy. 2011.