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REVIEW

BIOMARKERS OF EXPOSURE FOR ASSESSING ENVIRONMENTAL METAL POLLUTION: FROM MOLECULES TO ECOSYSTEMS

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ABSTRACT

Metals are among the most prevalent substances released into the environment that have a profound effect on living organisms. Chronic environmental exposures usually exert a continuum of biological responses across levels of biological organization, ranging from alterations in molecules, compromising individual health and putting ecosystem integrity at risk. Such scenarios have triggered the research to establish "early-warning" signals, or "biomarkers", reflecting the adverse biological responses towards environmental pollution. In this review, we assess the different types of biomarkers most used to analyze environmental metal pollution across all levels of biological organization and in each section representative examples in human and animal species and/or wild populations are given. Also, the "omics" approach is described and how these novel technologies are reinventing the field of toxicology, providing "molecular signatures" of exposure, enabling a more robust risk assessment than has ever been achieved previously. Finally, conclusions and suggestions are given, highlighting why future efforts must focus on integrating biomarker response across levels of biological organization, which integrate realistic exposures using multi-species and multiple-biomarkers with prognostic value to resolve or at least have a closer insight into complex environmental problems.

Palabras clave: metales, biomarcadores, marcadores ecológicos, genética toxicológica, ecotoxicología, tecnología genómica

RESUMEN

Los metales se incluyen dentro de las substancias más persistentes emitidas al ambiente, los cuales tiene efectos importantes sobre los seres vivos. La exposición ambiental crónica a los metales generalmente resulta en un continuo de respuestas biológicas que se da en todos los niveles de organización biológica. Estas respuestas pueden observarse desde alteraciones a nivel molecular, comprometiendo la salud del individuo, hasta poner en riesgo la salud del ecosistema. Lo anterior ha impulsado la

investigación científica para establecer "señales tempranas de alerta" mediante el uso de "biomarcadores", los cuales reflejen los efectos biológicos adversos producidos por los contaminantes ambientales. En este trabajo se revisan los biomarcadores más utilizados para estudiar la contaminación ambiental producida por metales, en todos los niveles de organización biológica y en cada sección se dan ejemplos representativos en humanos, especies animales y poblaciones silvestres. Además, se describe desde la perspectiva de las ciencias ómicas, como estas metodologías han reinventado el campo de la toxicología, proporcionando "huellas moleculares" de exposición, permitiendo así un análisis de riesgo más robusto el cual no se había alcanzado antes. Finalmente, se dan conclusiones y sugerencias resaltando la razones de por qué los esfuerzos futuros deben enfocarse en la integración de las respuestas proporcionadas por los biomarcadores en todos los niveles de organización biológica, que consideren exposiciones más apegadas a la realidad, mediante diseños experimentales más rigurosos utilizando multiespecies y multibiomarcadores con valor predictivo para resolver, o entender mejor los problemas ambientales complejos.

INTRODUCTION

The environment is continuously loaded with foreign chemical substances, released by anthropogenic activities. As a result, many wildlife and human populations are exposed to a variety of chemical agents which may lead to a collection of biological effects. Among environmental pollutants, metals have been identified among the most toxic elements to nearly all living organisms (EPA 2000). The relationship between metal toxicity and a plethora of effects is well established. Studies from populations exposed to metals, were among the first to establish quantitative relationships between the external exposure, the internal dose, and the early effects (Bernard 2008).

Organisms integrate exposure to contaminants in their environment and respond in some measurable and predictable way, being these responses observed and measurable across different levels of biological organization (Bickham *et al*. 2000). In the field of toxicology, it is essential to be able to measure the exposure to a toxic agent, the extent of any toxic response and also to predict the likely effects. Hence, integrating measures of different types of responses to toxic stress of exposed individuals and populations, offers a powerful tool for documenting the extent of exposure and the effects of environmental metal contamination. Tools that enable this to be done are called "biological markers" or "biomarkers". For these reasons, the use of biomarkers for environmental monitoring of individuals and populations exposed to chemical pollution has gained much attention in the last decades, because it offers great opportunities for a fast and sensitive detection of chemical stresses within organisms (Peakall and Shugart 1992, Handy *et al*. 2003).

The use of biomarkers in environmental health was described in a series of publications issued by the Board of Environmental Studies in Toxicology of the National Research Council (NRC 1987, 1989) of the USA. The NRC defines biomarkers as "Indicators of events in biological systems or samples" and was further described as "tools that can be used to clarify the relationship, if any, between exposure to a xenobiotic substance and disease". Also, the NRC classified biomarkers into three categories based on their relation to the exposure-disease continuum: biomarkers of exposure, effect and susceptibility. Some years later, Lagadic *et al*. (1994) referred to biomarkers as "biochemical sub-lethal changes resulting from individual exposure to xenobiotics". These definitions denote that many researches focus on biomarkers as measures at the cellular or sub-cellular levels, as in the case of molecular epidemiology and genetic toxicology, where measurements of toxic responses are routinely used to infer cause-effect relationships between biomarker response and health effects of the exposed individuals (Perera 2000). Also, the former definitions restrict the term biomarker to measurements at or below the level of individuals. Hence, it becomes important to consider that there are other types of biomarkers that attempt to measure effects of chemical pollution at the population, community and even at the ecosystem level (ecotoxicology). This reflects the fact that pollutants can exert their influence at all levels of biological organization (Lagadic *et al*. 1994, Peakall 1994). In this context, Handy *et al*. (2003) expanded the concept as "the identification of specific molecular, biochemical, physiological and behavioral changes in populations following pollutant exposure". Both approaches try to reveal cause-effect relationships between the initial exposure and the subsequent effects, based on the use

of biomarkers, but in different levels of biological organization.

In this review, we assess the most common biomarkers used in each level of biological organization. The first section, deals with biological responses exerted by metals from molecules to individuals. The next section, addresses biological responses from populations to ecosystems. In each section, representative examples concerning environmental metal exposures in humans and animal species (individuals and populations) are given, in order to illustrate how the use of biomarkers is suitable for studying metal exposures.

Also, a new approach is described, the "omics" approach, where the search for new biomarkers becomes possible. These novel technologies offer added value compared with classical testing with whole organisms because they provide information concerning the molecular basis of exposure "molecular signatures" and act as "early warning" signals, enabling a more robust environmental monitoring than has ever been achieved previously (Snape *et al.* 2004).

DEFINITIONS AND TYPES OF BIOMARKERS: FROM MOLECULES TO INDIVIDUALS

Many metals are essential to living organisms but some of them are highly toxic or become toxic at high concentrations, these include iron (Fe), Copper (Cu), Zinc (Zn), Cobalt (Co), Molybdenum (Mo), and Manganese (Mn). Light metals such as Sodium (Na), Potassium (K), and Calcium (Ca) play important biological roles. Metals such as Mercury (Hg), Lead (Pb), Niquel (Ni), Chromium (Cr), Cadmium (Cd), and Arsenic (As) are generally not required for metabolic activity and are toxic to living organisms at quite low concentrations (Valavanidis and Vlachogianni 2010). Other metals such as Vanadium (V) which is present in almost all-living organisms but its essentiality in cellular functions is yet to be established, is also capable of inducing toxic effects in various species (Rodríguez-Mercado and Altamirano-Lozano 2006). As a consequence, the toxicological effects of metals have been widely studied, where it has been recognized that the relationship between exposure and disease is as a multistage process which includes external exposure, internal dose, early biological effects, altered structure and function and finally clinical changes or disease (Link *et al*. 1995, Vanden-Heuvel and Davis 1999).

When characterizing toxicological responses, it is desirable to distinguish each step in this continuum. Biomarkers signify these alterations in biological systems and may be indicators of exposure, effect or susceptibility and may overlap sometimes (Perera 1996, Perera and Weinstein 2000, Jakubowski and Trzcinka-Ochoka 2005, Nordberg 2010).

*Biomarkers of exposure***:** "An exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that it is measured in a compartment within an organism". These types of biomarkers are also known as "biological dosimeters" or biomarkers of internal dose, and when they measure the product of the interaction with target molecules they are regarded as "biomarkers of biological effective dose" (Timbrell 1998).

*Biomarkers of effect: "*A measurable biochemical, genetic, physiological, behavioral or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or early health impairment or disease" (Timbrell 1998).

Biomarkers of susceptibility: "An indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance" (Pavanello and Clonfero 2000, Sakai 2000).

TYPES OF BIOMARKERS OF EXPOSURE

Biomarkers of internal dose: these are the most used, because of their precision, reliability and relevance to individual risk (Perera and Weinstein 2000, Aitio *et al*. 2007, Nordberg 2010). They have been used in combination with measures of external exposure. Currently, highly sensitive analytical methodologies make possible to measure very low concentrations of a chemical substance or its metabolite in various cell types, organs or body fluids. These types of biomarkers take into account individual differences in absorption, metabolism, bioaccumulation and excretion of the compound in question and indicate the actual dose of the substance within an organism and in specific tissues (Perera and Weinstein 2000).

Examples of internal dosimeters of metal exposure include: hair, nail, blood, and urinary levels of total inorganic As or its metabolites (Hughes 2006, Fowler *et al*. 2007), Pb blood concentrations (Bjorkman *et al*. 2000, Aitio *et al*. 2007), Cd blood, urine and kid-

ney concentrations (Clarkson *et al*. 1988, Nordberg *et al*. 2007, Nordberg 2010) and methylmercury in hair (Jakubowski and Trzcinka-Ochocka 2005) and V concentrations in kidney and liver (Gummow *et al*. 2006). For a more detailed review and examples see Fowler (1987, 1992).

Although biomarkers of internal dose are a valuable tool for assessing chemical exposures, they do not indicate the extent to which a given compound has interacted with molecular and cellular targets. For this reason assays have been developed to measure the "biological effective dose" (Perera and Weinstein 2000).

*Biomarkers of biological effective dose***:** These types of biomarkers occur early in the exposure-to-disease pathway. Some of them have been shown to be associated with increased risk of developing diseases such as cancer. As a result, they are considered as important tools for investigating mechanisms behind exposure-induced adverse health effects (Perera 2000, Sorensen *et al.* 2003). The best known examples are DNA-adducts.

The study of DNA-adducts is motivated by the fact that many environmental contaminants and some metals are thought to exert their genotoxic effects through covalent binding with DNA (Perera and Weinstein *et al*. 2000, Poirier 2004, Gallo *et al*. 2008). DNA-adducts are addition products formed by covalent binding of all or part of a metal molecule to chemical moieties in DNA; adducts are formed when an activated chemical species (electrophilic, positively charged metabolite) binds covalently to negatively charged moieties. In other words, they represent the amount of a given metal that has reacted with critical cellular macromolecules such as DNA or proteins in a given tissue (Ehrenberg *et al*. 1996). In this context, DNA-adducts are among the most informative biomarkers of exposure to genotoxic agents (Poirier 2004).

The quantification of DNA-adducts gives information about the biologically effective dose of a metal reaching the DNA in cells. As a result, they represent the amount of the metal that has been absorbed by the body, undergone metabolic activation, become bound to cellular DNA and has not been repaired (Rundle *et al*. 2002, Gallo *et al*. 2008). DNA-adducts if not repaired or repaired inadequately, may lead to mutation and alteration of gene function (Farmer 2004, Jakubowski and Trzcinka-Ochocka 2005, Swenberg *et al*. 2008).

Early studies utilized column chromatography to examine adduct formation, but this technique has a detection limit of 1 adduct per 10^6 nucleotides (Swenberg *et al*. 2008). Thereafter, Randerath *et al*. (1994) developed one of the most used techniques to analyze the extent of DNA-adducts, which is ^{32}P-postlabelling, detecting at least one adduct per 10^8 nucleotides. Recently, using accelerator mass spectrometry, 1 adduct per 10^{12} bases are possible to detect, which is probably 1 adduct per cell (Singh and Farmer 2006, Swenberg *et al*. 2008). It is important to mention that although the formation of DNA-adducts is not the main mechanism of toxicity of metals, many authors mention that they may form DNA-adducts directly, as in the case of Cr (Singh *et al*. 1998, Zhitkovich 2005) and water soluble Ni compounds (Muller *et al*. 1999), and indirectly (through formation of free radicals and reactive oxygen species (ROS)) as in the case of As (Wang *et al*. 2001, Bau *et al*. 2002, Rossman 2003, Méndez-Gómez *et al*. 2008).

TYPES OF BIOMARKERS OF EFFECT

Biomarkers of effect are perhaps best regarded as indicators of early changes that could later lead to clinical disease (Mutti 1995). There are situations where biomarkers of exposure are not sufficient to predict potential adverse effects. In such situations, biomarkers of effect are used to understand if a change in their distribution has occurred as a result to the chemical exposure. Hence, biomarkers of effect are not proof of disease caused by environmental pollution but tools to understand a process that might eventually lead to adverse effects (Watson and Mutti 2004). Biomarkers of effect give measures of the alterations on important genetic targets like DNA, causing DNA-breaks, chromosome aberrations and micronucleus. Biomarkers of biochemical effect provide information about oxidative damage in DNA and proteins, alterations in a wide range of enzymes like DNA-repair enzymes, and metal-binding proteins, among others (Frenzilli *et al*. 2009, Rojas 2009).

*DNA single (SSB) and double strand breaks (DSB)***:** Another approach for evaluating the possible consequences of environmental metal pollution involves the assessment of genotoxic damage measured as DNA-breaks. Most metals interact indirectly with DNA, via generation of ROS, causing single and double strand breaks (Valko *et al*. 2006, Mussali-Galante *et al*. 2007, Frenzilli *et al*. 2009).

The DNA molecule must undergo continuous maintenance to sustain its integrity. Several key mechanisms in DNA-repair processes involve the

degradation of a short stretch of DNA leading to a transitory break in a single DNA strand. The incidence of such strand breaks may be enhanced both as a direct result of metal exposure or as an indirect effect of repair processes (Shugart and Theodorakis 1994, Hebert and Murdoch 1996). Measurement of DNA-breaks induced by metal exposure is common there are several approaches to quantify the frequency of DNA strand breakage: the alkaline unwinding assay (Shugart 1988), the single cell gel electrophoresis assay or "comet assay" (Rojas *et al*. 1999), chromosomal aberrations (Obe *et al.* 2002), alkaline elution (Koch and Giandomenico 1994) and sister chromatid exchanges (Perry and Wolf 1974), among others (Ahnstrom 1988, Lindberg *et al*. 2007).

The comet assay, is a rapid, simple, and sensitive technique for analyzing DNA breakage of single and double strands, depending of pH conditions, in individual cells (Singh *et al*. 1988, Silva *et al*. 2000, Tice *et al*. 2000, Mussali-Galante *et al*. 2005, Rojas 2009). In principle, any organism is suitable for the comet assay and small cell samples are needed. As a result, the comet assay has become one of the major tools for environmental biomonitoring studies (Valverde and Rojas 2009). It is important to mention that even when the Comet assay is sensitive to detect strand breaks, it is a nonspecific chemical biomarker of genotoxicity (Dhawan *et al*. 2009). However, the versatility in terms of cell types used to determine DNA-breaks as a consequence of metal exposure is illustrated in the following examples: Humans exposed to As (Abernathy *et al*. 1999, Calderón *et al*. 2003, Pandey *et al*. 2007), humans exposed to V (Ehrlich *et al*. 2008), coelomocytes exposed to Ni (Reinecke and Reinecke 2004), earthworms exposed to Cd (Fourie *et al*. 2007), grasshoppers exposed to Zn (Augustyniak *et al*. 2006), birds (Baos *et al*. 2006, Pastor *et al*. 2001, 2004), mussels (Machella *et al*. 2006) and wild mice species exposed to heavy metal mixtures (Leon *et al*. 2007, Tovar-Sánchez *et al*. 2012). More detailed examples are given by Dhawan *et al*. (2009) and Frenzilli *et al*. (2009).

Chromosome aberrations (CA): From the vast scientific literature assessing CA as a biomarker of effect, it is evident that cytogenetic biomarkers have been a valuable tool for studying the most important environmental hazards occurring in the past decades. The use of valid biomarkers of risk in populations exposed to genotoxic agents is the most suitable and well-established approach for analyzing many modern exposures (Tucker and Preston 1996, Bonassi *et al*. 2005). CA are induced by agents that damage chromosomal DNA (Natarajan 1976). A large amount of evidence demonstrates that DNA-DSB are the principal lesions in the process of CA formation (Pfeiffer *et al*. 2000). DSB arise spontaneously at high frequencies through a variety of cellular processes (Natarajan 1976, Bonassi *et al*. 2005). However, the majority of chemical mutagens are not able to induce DSB directly but lead to other lesions in chromosomal DNA which, during repair or DNA synthesis, may give rise to DSB and eventually to CA (Tucker and Preston 1996, Obe *et al*. 2002).

Many studies assessing the frequency of CA and other genotoxic endpoints resulting for environmental metal exposure have been conducted in As exposed human populations (Ostrosky-Wegman *et al*. 1991, Gonsebatt *et al*. 1997) and in animal species inhabiting superfund sites. In this context, the studies of McBee *et al*. (1987) and McBee and Bickham (1988) where the first to report higher levels of karyological damage in two wild rodent species living in a metal contaminated site. Also in many fish species (Prein *et al*. 1978, Hooftman and Vink 1981) in orthopterans (*Tetrix tenuicornis)* living in zinc-lead mine spoils (Warchałowska-Śliwa *et al*. 2005), and in dipterans (*Chironomus riparius*) inhabiting a polluted site (Zn, Cd, Pb, Cu) (Michailova *et al*. 1996).

It is important to mention that unlike chemical DNA-adducts, chromosomal aberrations are a non-chemical specific biomarker (Perera 2000).

Micronuclei (MN): As their name suggests, micronuclei are masses of DNA (resembling small nuclei) found in the cytoplasm, rather than being contained within the nuclear membrane. Micronuclei form when acentric or centromeric chromosome fragments are unable to attach to a spindle fiber during cell division or when an intact chromosome is excluded from the nucleus because of defective cell division. Hence, micronuclei may be a consequence of either chromosomal breakage or dysfunction of the spindle mechanism (Lindberg *et al*. 2007). These types of micronuclei can be distinguished (Boei and Natarajan 1995), and there is evidence that genotoxic agents can be differentiated by whether they induce chromosomal breakage or loss (Chen *et al*. 1994, Fenech and Crott 2002) and/or centromeric modifications (Fenech *et al.* 1999). They have been studied for many years, in experimental research as well as in environmental monitoring. In the last decade, MN assay has gained a lot of attention because it offers several advantages: a) MN can be observed in almost any eukaryotic cell type, b) Speed, ease and low cost of the analysis, and c) the non-requirement for me-

taphase cells. Thus, MN analyses can be employed in studies with different experimental conditions, in a wide variety of animal species (Bonassi *et al*. 2005). For many years, research employing the MN assay in environmental exposures has been conducted in individuals exposed to As in drinking water (Fenech *et al*. 1999, Basu *et al*. 2004).

Recently, research has been carried out to evaluate the clastogenic and/or aneugenic activity of different environmental metal pollutants in natural animal populations (Bolognesi and Hayashi 2011). For this purpose, the fish erythrocyte micronucleus test has been used as an informative biomarker to evaluate the clastogenic potential of metals in water (Al-Sabti 1994, Minissi *et al*. 1996, Russo *et al*. 2004). Other examples that report statistically high frequencies of MN include, eels (*Anguilla anguilla*) exposed to Cd and Hg (Sánchez-Galán *et al*. 2001), and the wood mouse (*Apodemus silvaticus*) exposed to Cd, Fe, Zn, Cu, Mn, Mo and Cr (Sánchez-Chardi *et al*. 2007).

Sister chromatid exchange (SCE): This assay is a well-known cytogenetic technique that has been used extensively to assess DNA damage at the chromosomal level (Hagmar *et al*. 1994). SCE occur as a normal feature of cell division in mammalian cells. They are believed to represent the interchange of DNA replication products at apparently homologous loci which involve DNA breakage and reunion (Gauthier *et al*. 1999, Wilson and Thompson 2007). During the S-phase of the cell cycle, DNA is replicated, and each chromosome becomes duplicated into two closely associated daughter chromatids that are linked tightly at the centromere. Sister chromatids are visible cytologically in late prophase and early metaphase of mitosis before chromosome segregation occurs (Latt 1973, Kaina 2004). Hence, SCE is the process whereby the sister chromatids effectively break and rejoin with one another, physically exchanging regions (Kato 1974, Perry and Wolf 1974, Wilson and Thompson 2007). While SCE are readily observed experimentally, the mechanisms that mediate SCE are not fully understood and controversial results have been reported (Ohno *et al*. 1982, Hartmann and Speit 1994, Fogu *et al*. 2000, Wilson and Thompson 2007, Tapisso *et al*. 2009). Particular types of genotoxic chemicals like bifunctional alkylating agents are in general potent inducers of SCE, presumably because homologous recombination is required to repair the resulting broken replication forks that arise during crosslink releasing (Thompson 2005). Metals that are known to induce SCE are cadmium, chromium, aluminum, arsenic, lead, vanadium and zinc (Sivikova and Dianovsky 1995, Bilban 1998, Mouron *et al*. 2004). This evidence comes mainly from *in vitro* (Fan *et al*. 1996, Basu *et al*. 2001, Rodriguez-Mercado *et al*. 2003, Mouron *et al*. 2004) and *in vivo* (Mukherjee *et al*. 1988, Gennart *et al*. 1993, Lai *et al*. 1998, Tapisso *et al*. 2009) studies or from studies of humans exposed to arsenic in drinking water (Ostrosky *et al*. 1991, Lerda 1994, Basu *et al*. 2001, Rossman 2003). However, there are very few studies assessing the induction of SCE in wild animal populations [Arctic beluga whale (*Delphinapterus leucas*), Gauthier *et al*. 1999] exposed to environmental metal stress in comparison with other biomarkers of early effect. Since these biomarkers (SSB, DSB, MN, CA) analyze different types of DNA damage, which can have dissimilar sensitivities to metals, these assays should be used complementary, along with the inclusion of SCE for biomonitoring exposure to genotoxic compounds in the natural habitat of different animal populations.

BIOMARKERS OF BIOCHEMICAL EFFECT

*Oxidative damage***:** Under normal physiological conditions in all aerobic organisms, there is a balance maintained between endogenous oxidants and numerous enzymatic and non-enzymatic antioxidant defenses (Halliwell and Gutteridge 1999). When an imbalance occurs, oxidants produce extensive oxidative damage to macromolecules such as DNA, proteins and lipids, which, in turn, contributes to aging, cancer, and other degenerative diseases.

Nearly 100 different oxidative DNA modifications have been identified, ranging from modified bases to DNA-breaks in a wide variety of animals and human cells exposed to chemical agents (Dizdaroglu 1992, Cadet *et al*. 2002). In all cells, altered DNA is repaired enzymatically, while misrepaired DNA can result in mutations leading to genomic instability and cancer (Kawanishi *et al*. 2001). Although a broad range of DNA alterations are produced during oxidative damage to DNA, most interest has focused on guanine oxidation products, among them are, 8-hydroxyguanine (8-oxo-G), 8- hydroxyguanosine (8-oxyGuo) and 8-hydroxy-2-deoxyguanosine (8-OHdG). One of the most abundant lesions is 8-OHdG, which is formed *in vivo* and can be measured quantitatively in cells following hydrolysis of the DNA to component bases (Valavanidis *et al*. 2009). This lesion is a major product of hydroxyl radical attack on DNA and of maximum biological importance. Also, 8-OHdG has attracted particular attention because it causes

G-to-T transversions and its presence may lead to mutagenesis (Hayes 1997, Wong *et al*. 2005, Valavanidis *et al*. 2009). Measurements of 8-OHdG, or its corresponding nucleoside, after repair processes results in the excised 8-OHdG adduct being excreted in urine, and because of its easy collection, these biomarkers are among the most widely used markers of oxidative DNA damage (Wong *et al*. 2005). The 8-OHdG level in DNA isolated from tissue is believed to exemplify the steady state damage of DNA being a result of damage and repair, while 8-OHdG excreted in urine is alleged to be an indicator of total DNA excision repair within an organism. As it is assumed that DNA repair under normal conditions is almost complete, 8-OHdG excretion is also a marker of the rate of total DNA damage (Loft and Poulsen 1999, Sorensen *et al*. 2003).

Because of its capacity to lose electrons, a metal is primarily thought to be toxic by virtue of its generation of ROS. Thus, exposure to high concentrations of a single heavy metal might result in its accumulation and potentially, oxidative damage (Limón-Pacheco and Gonsebatt 2009). Metals such as Fe, Mn, Ni, Cu, Cr, and V can generate ROS in biological systems causing oxidative damage in DNA and proteins (De Flora and Wetterhahn 1989, Gurgueira *et al*. 2002, Valavanidis *et al*. 2005, 2009, Valko *et al*. 2005). Specifically, the induction of 8-OHdG has been reported after *in vivo* exposure to As (Rossman 2003), Cd (Filipic and Hei 2004), Co (II) (Mao *et al*. 1996), Cr (VI) (Kuo *et al*. 2003), and V (Shi *et al.* 1996, Rodríguez-Mercado *et al.* 2003).

DNA repair enzymes: A general mechanism of carcinogenicity of As, Cd, Co, and Ni seems to be the inhibition of DNA repair enzymes and the consequent enhancement of DNA damage originally caused by other agents or raised spontaneously (Beyersmann 2002). Even though, the inhibition of DNA repair processes appears to be a common mechanism of action of some metal compounds, the steps affected seem to be rather different. One mechanism of repair inhibition is the displacement of essential metal ions such as Zn, Mn, Ni, and Co (Hartwig *et al*. 2002, Rossman 2003).

Some toxic metal ions have high affinities toward sulfhydryl (SH) groups, as a result, potential targets are the so called "zinc finger proteins". Although most zinc finger structures have been described as DNA-binding motifs in transcription factors, they have also been identified in several DNA repair enzymes (Rossman 2003). They include the mammalian XPA protein, the bacterial Fpg protein and the poly (ADP-ribose) polymerase.

Specifically, the Fpg protein is inhibited by Cd, Cu, and Hg and Ni and Co inhibit DNA binding of XPA (Asmuss *et al*. 2000). Also, poly (ADP-ribose) polymerase is inhibited by arsenite in mammalian cells (Hartwig *et al*. 2002, Schoen *et al*. 2004). For more comprehensive examples and molecular mechanisms see Hartwig *et al*. (1997) and Hartwig (1998, 2001).

These proteins have been used as biomarkers to analyze response to toxic metals. These findings have been observed at low concentrations, in most cases more than ten-fold below the cytotoxic level. Thus, under environmental exposure conditions, repair inhibition may contribute significantly to metal-induced toxicity and carcinogenicity (Méndez-Gómez *et al*. 2008). However, environmental exposures analyzing alterations in repair enzymes are scarce because the difficulty to link specific enzyme alterations exclusively to metal exposure.

Metallothioneins: For metals, much of the work in the area of biomarkers has focused on metallothioneins or metallothionein-like proteins (MT). These low-molecular weight, cysteine-rich metal-binding proteins are reported to play a key role in the binding and transport of various metals (Costa *et al*. 2008, 2009). The structure of these highly conserved proteins is linked to their role in the homeostasis of essential metals such as Zn and Cu and detoxification of toxic elements such as Cd and Hg. MT have several isoforms, apparently induced by different metals, the best known of which, MT-I and MT-II, are greatly induced by Cd and Zn (Viarengo *et al*. 1999, Romero-Isart and Vasak 2002).

MT induction is considered as a biochemical biomarker of exposure and of biologically effective dose, and can be used to point trace metal environmental exposures (Langston *et al*. 1998, Olsvik *et al*. 2001).

Another possible use of MT as a biomarker, involves the examination of the intracellular distribution of metals among cytosolic ligands, including MT. These types of changes offer several advantages as biomarkers; since molecular alterations are normally the first detectable, it becomes possible to quantify early responses to environmental metal stress. As a result, some authors have suggested that they may serve as markers of both exposure and effect (George and Olsson 1994, Olsvik *et al*. 2001). Hence, their use in environmental metal monitoring surveys has been well established (Perceval *et al*. 2004).

There is a considerable amount of literature concerning MT induction following metal envi-

ronmental exposure. Most of the studies have been conducted in humans and in aquatic animals. Some representative examples include: MT levels in liver and kidney of Canadian individuals exposed to Cd and Zn (Chung *et al.* 1986), MT induction in peripheral lymphocytes from Chinese individuals exposed to Cd (Lu *et al*. 2005), brown trout (*Salmo trutta*) exposed to Zn, Cd, Cu (Olsvik *et al*. 2001), the great tit (*Parus major*), along a metal pollution gradient (Pb, Cd) (Vanparys *et al*. 2008), and the fish (*Solea senegalensis*) exposed to As, Cd, Cr, Cu, Ni, Pb and Zn (Costa *et al.* 2009) and mussels (*Mytillus* sp.) exposed to V from an oil spill (Amiard *et al.* 2008). All these studies conclude that MT are modulated by heavy metals, being an informative and specific biomarker of chronic heavy metal exposure. More examples are reviewed in Petering and Fowler (1986), Nordberg (1998).

Aminolevulinic acid dehydratase (ALAD): It is well known that individuals exposed to lead may develop anemia, mainly from the interaction of lead with some enzymatic processes responsible for heme synthesis, like the inhibition of ALAD. ALAD is the second enzyme in the heme biosynthetic pathway which catalyses the condensation of two molecules of aminolevulinic acid to form one molecule of porphobilinogen. Erythrocyte ALAD activity is rapidly inhibited by lead exposure (Sakai *et al*. 1981). Therefore, determination of ALAD activity in erythrocytes is one of the most useful and well established biomakers for evaluating lead exposure, because the activity is extremely sensitive to and specific for blood lead concentration. If ALAD is inhibited, it is a clear indication of the presence of biological significant quantities of lead, but measurements of the activity of ALAD do not provide information on the presence of any other pollutants (Sakai *et al*. 1996, Sakai and Morita 1996, Sakai 2000). ALAD activity has been frequently measured in human adult individuals and children, as well as in animals after Pb environmental exposures were detected. For example, ALAD activity was significantly lower in a population of Indian children with the highest lead blood levels when compared to children with medium and low lead blood levels (Ahamed *et al*. 2005). Similar results were obtained among urban adolescents (Ahamed *et al*. 2006) and among adults and elderly people (Todd *et al*. 1996, Lee *et al*. 2006). In animals, ALAD activity has been frequently measured in birds (Johnson *et al*. 1999, Strom *et al*. 2002, Beyer *et al*. 2004, Vanparys *et al.* 2008), amphibians (Arrieta *et al*. 2004) and tortoises (Martinez *et al*. 2010).

BIOMARKERS OF SUSCEPTIBILITY

Given the fundamental role of metabolism in toxicological research, increasing attention in the role of genetic variation in toxic responses, and therefore variations in susceptibility and markers of such susceptibility, are of great interest (Timbrell 1998). Initial biomarker research into host factors has been directed at the identification of inter-individual differences in metabolic pathways. A wide range of enzymes that may be associated with disease have been explored, demonstrating substantial differences in levels of activity within the population, such as N-acetyltransferase, several cytochromes P-450 (CYP), and glutathione transferase (GST), among others (Cullen and Redlich 1995, Timbrell 1998, Pavanello and Clonfero 2000). Specifically, trace metals are reported to regulate the expression of CYP as well as heavy metals like Hg and Pb (Ki *et al*. 2009). Each of these enzymes has a potential role in the activation or detoxification of chemical exposures. As the genetic loci of these and other metabolic enzymes have been recognized, the identification of polymorphisms and phenotypic differences in the population has become possible. Polymorphisms and/or acquired differences in enzyme function might be, in part, the cause for differential responses to metals (Cullen and Redlich 1995). As a consequence, the study and identification of single nucleotide polymorphisms (SNPs), becomes essential when studying responses to metal exposures. SNPs are the most abundant forms of DNA sequence variation in the human genome, and contribute to phenotypic diversity, influencing risk of certain diseases, and variable response to the environment (Pavanello and Clonfero 2000).

In the last 20 years, many research groups have been involved on assessing the genotoxic risk of exposed populations according to their genetically determined metabolic characteristics (Timbrell 1998, Pavanello and Clonfero 2000). Unfortunately, in humans most susceptibility studies have focused on infectious diseases or risk factors for cardiovascular disease or cancer and very little attention has been devoted to susceptibility to metal toxicity. Among the few examples analyzing the influence of SNPs on metal exposure responses are: Gundacker *et al*. (2007) analyzing the relationship between polymorphisms in GST genes in individuals exposed to Hg. Also, Tekin *et al*. (2012) determined MT polymorphism in pregnant woman and lead blood levels, concluding that enzyme polymorphisms are well correlated with metal concentrations and with individual susceptibility to toxic effects of metals. For detailed review

TABLE I. DIFFERENCES BETWEEN BIOMARKER RESEARCH APPROACHES FOR ASSESSING ENVIRONMENTAL METAL POLLUTION

Molecules to individuals	Individuals to ecosystems
Usually single compounds	Complex mixtures
High doses, acute exposures	Low doses, chronic exposures
Animal models or occupationally exposed populations	Sentinel species, natural populations
Biomarkers at lower levels of biological organization	Biomarkers at higher levels of biological organization, considering responses at lower levels
Usually non-neutral markers	Usually neutral markers
Concerned with individual and population susceptibility	Concerned with population and ecosystem health
Mechanistic importance	Ecological importance
Time scale decreases	Time scale increases

of MT polimorphisms as biomarkers of individual susceptibility, see Nordberg (1998).

Studies with inorganic arsenic have contributed to a great extent to the knowledge of differences in metal exposure metabolism-responses. Studies concerning the effects of polymorphic forms of arsenic methyl-transferase (AsMT) in regulating the toxicity of AsIII in mice (Stýblo *et al*. 2002, Aposhian *et al*. 2004, Wang *et al*. 2008) highlighted the importance of polymorphisms in the metabolic pathway in mediating formation of toxic methylated arsenical metabolites.

In relation to the susceptibility of lead effect on heme metabolism, several groups have investigated the relationships between ALAD polymorphism and susceptibility to lead toxicity (Schwartz *et al*. 1995, Sakai *et al*. 1996, Alexander *et al*. 1998). These studies concluded that ALAD1 homozygotes might be more susceptible for disturbance in heme biosynthesis than ALAD2 carriers, supported by the fact that ALAD2 protein may bind lead more tightly than ALAD1 protein.

In general, differences in response to heavy metal-associated effects based on genetic variability are not well understood. The only genetic background is far better known for arsenic, mercury and lead, than for the rest of the metals (Gundacker *et al*. 2010).

One reason for the scarce literature on susceptibility of metals is the difficulty of measuring it in isolation; it cannot be separated from other exposures, and controlled exposures are seldom used in humans and difficult to find in natural animal populations.

DEFINITIONS AND TYPES OF BIOMARKERS: FROM INDIVIDUALS TO ECOSYSTEMS

Until now, we have observed that the use of biomarkers at the cellular or sub-cellular levels for analyzing environmental metal exposure is adequate, useful and in some cases, well established. For many years, studies in genetic toxicology and molecular epidemiology have focused on the effects of acute exposures to single toxicants at high doses. Therefore, such biomarkers contribute little to the prediction of the direct consequences for the population in question, hence to the community and ecosystem health. On the contrary, in ecotoxicology threats to populations and communities rising from chronic exposures to mixtures of chemical agents at lower doses (realistic exposures) are the point of interest (Depledge 1994) (**Table I**). Hence, establishing links between cellular and sub-cellular effects and their possible consequences at higher levels of biological organization becomes essential. This is possible by the use of biomarkers in each level of biological organization (**Fig. 1**).

However, incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Definition of biomarkers for ecotoxicology should expand the concept to include changes at the population, community and ecosystem levels, since chemical agents exert their effects at all levels of biological organization. Many studies from the ecotoxicological point of view refer to responses to toxic effects as "*Ecological indicators*" (Cairns and McCormick 1992, Hunsaker 1993). In many other cases, the same responses are regarded as "Biomarkers at population and community levels" where shifts in population and community parameters due to chemical pollution are included (Fossi 1994, Depledge and Fossi 1994, Evenden and Depledge 1997, Moore *et al*. 2004, Bernard 2008). At this point, biomarkers or ecological indicators should give additional information that cannot be obtained from chemical analysis of pollutant concentrations alone, and they may integrate effects of mixtures of chemicals over long exposure periods (Handy *et al*. 2003).

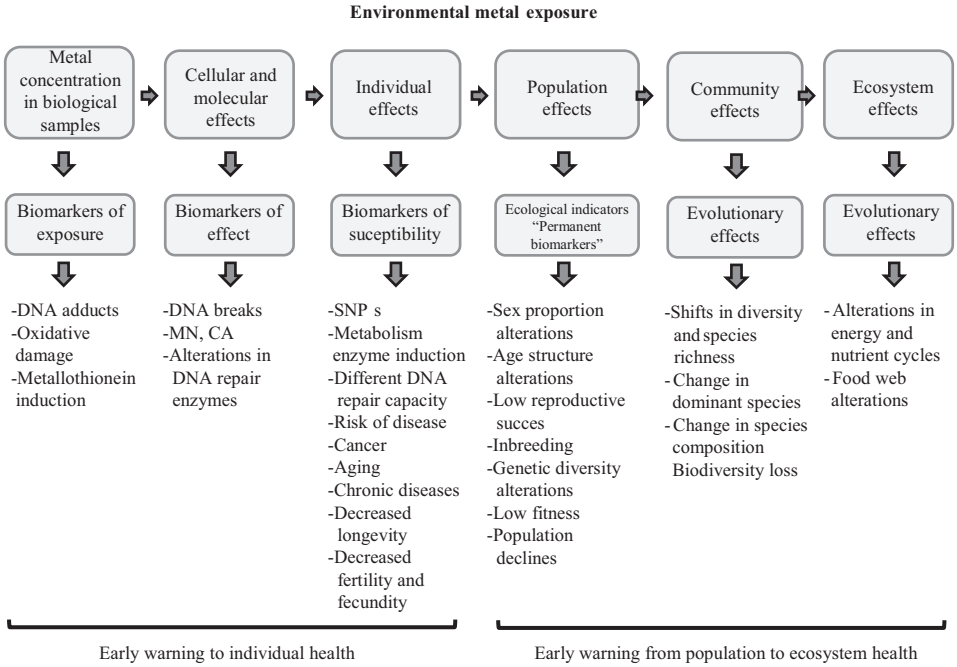

Fig. 1. Environmental pollutants –such as metals– can exert their effects at all levels of biological organization. Most used biomarkers for assessing toxic responses are listed in each level. MN= micronuclei, CA= chromosome aberrations, SNPs= single nucleotide polymorphisms.

Peakall (1994) suggested that when integrating biomarkers to ecotoxicology, three assumptions must be taken into account, since responses to chemical stress from the molecular to the ecosystem level is a continuum of events.

First, the timescale increases, moving from seconds or minutes to years or even decades. Second, the ecological importance increases. Third, it becomes difficult to relate effects to causes as one move up to this continuum (because specific biomarkers for a given chemical agent are more difficult to find). In our opinion, another assumption needs to be taken into account: Mechanistic information about the modes of action of chemical agents is inferred in the lowest levels from this continuum (**Table I**, **Fig. 2**). Additionally, biomarkers should be chosen so that they reflect changes in the fitness of the population (premature death, ability to mate, fecundity, viability of offspring, etc.) (Evenden and Depledge 1997).

Population level biomarkers

Large phenotypic shifts can evolve in populations over a short period of time. For example, large and rapid evolutionary changes (microevolution) are evident from population responses to pollutants

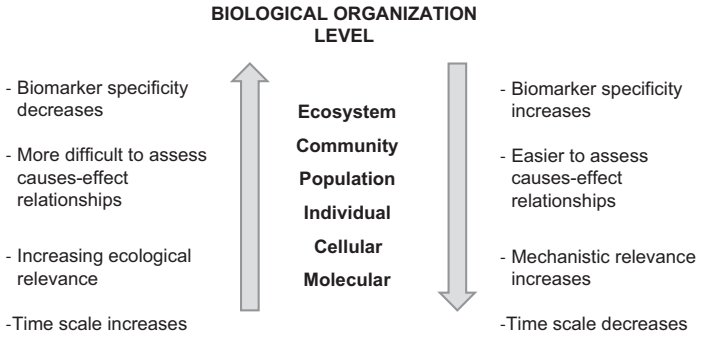

Fig. 2. Assumptions that need to be taken into account when using biomarkers to infer environmental pollution effects at different levels of biological organization. Arrows represent directionality at each level of biological organization for each assumption.

and chemical stress (Luoma 1977). Underlying these microevolutionary changes are shifts in allele frequencies at loci. These changes have long been considered as having potential for monitoring environmental stress.

This process was defined by Medina *et al*. (2007) as "Microevolution due to pollution", it occurs rapidly, in years or after few generations instead of centuries or millennia, involving a variety of physiological, morphological and life-history traits. This fact makes possible to use microevolutionary changes as biomarkers for assessing effects of chemical pollution at the population level (Hoffman and Dabron 2007, Mussali-Galante *et al*. 2012).

Among studies that deal with metal stress populations, the most used approach is to address changes in genetic diversity and allele frequency patterns by using neutral molecular biomarkers (Bickham *et al.* 2000). A neutral biomarker is a sequence of DNA that is polymorphic within a population or a species and that is not under selection. They play an important role in estimating the genetic diversity among individuals by comparing the genotypes at a number of polymorphic loci (Arif and Khan 2009). These markers inform about population demographic processes and have the potential to measure shifts in population size arising from environmental change and adaptation (Bickham and Smolen 1994, Harper-Arabie *et al*. 2004). This fact has led to the hypothesis that neutral markers could be used to monitor pollution effects in populations (Medina *et al*. 2007).

A number of neutral biomarkers which include nuclear and mitochondrial DNA (mtDNA) analyses, such as allozymes, RFLPs (Restriction Fragment Length Polymorphism), SSRs (Simple Sequence Repeats or microsatellite markers), RAPDs (Random Amplified Polymorphic DNA), DNA sequencing of mtDNA, and AFLPs (Amplified Fragment Length Polymorphism) are available with application to genetic ecotoxicological research. However, in the last decade the most used biomarkers to assess genetic diversity in animal populations exposed to metal pollution are: Allozymes, SSR's and mtDNA-sequencing.

Allozyme analysis: This is one of the oldest techniques to assess genetic variability in natural populations. This method analyzes electrophoretic shifts in the charge characteristics of enzymatic proteins produced by amino-acid substitutions. The majority of allozymes show co-dominant inheritance, and the variants are attributed to nucleotide substitutions causing charged amino-acid replacement. This technique detects one-third of amino acid substitution. However, the generally low level of polymorphism at allozyme loci often limits their resolving power in detecting population differences (Diamond *et al*. 1989). Despite their limited resolution, allozyme analysis remains the simplest and most rapid technique for surveying genetic diversity in single copy nuclear genes (Bickham *et al*. 2000). For example, Maes *et al*. (2005) used allozymes and SSRs to analyze allele and genotypic frequencies, levels of polymorphisms and heterozygosity in the European eel (*Anguilla anguilla*) exposed to a mixture of metals (Hg, Cd, Pb, Ni, Cr, As, Se, Cu, Zn). They reported a negative correlation between the level of bioaccumulation and allozymatic multi-locus heterozygosity. Hence, an individual's enzymatic heterozygosity seems to play an important role in the potential to counteract pollutant bioaccumulation. Also, Benton *et al*. (2000) observed decreased heterozygosity in the snail (*Pleurocera canaliculatum*) exposed to Hg using allozymes as biomarkers, reinforcing the use of allozyme analysis as a marker of contamination and possible selection for pollution resistance.

Microsatellites (SSRs): Broadly used for genetic structure and variability analyses, these are short tandem repeats of mono-to tetra-nucleotide repeats which are assumed to be randomly distributed through out the nuclear and mitochondrial genomes. SSRs detects length variation that results from changes in the number of repeat units and their mode of inheritance is co-dominant. Mutations in SSRs are high compared to other DNA markers, therefore, they are considered one of the best molecular markers (Yauk and Quinn 1996, Athrey *et al*. 2007, Tremblay *et al*. 2008) to analyze genetic variability within and between populations. Unfortunately, the identification of SSRs is expensive and requires cloning and sequencing, whilst SSRs primer pairs appear to be species-specific, cross species amplification has been revealed although reduced variability has been observed.

A study conducted by Athrey *et al*. (2007) in which selection for Cd resistance in the least killifish (*Heterandria formosa*) led to increased levels of resistance, but also a decrease in genetic variation as measured by microsatellites. Also, Bourret *et al*. (2008) using SSRs showed that chronic exposure to metal contamination (Cd, Cu) have impacted genetic diversity among populations of the wild yellow perch (*Perca flavescens*), which may affect the capacity of populations to respond to environmental changes. Similar results were obtained by Ungherese *et al*. (2010) who observed decreased genetic diversity in

Hg exposed populations of the sandhopper (*Talitrus saltator*), and by Mussali-Galante *et al.* (2012) in the small mammal (*Peromyscus melanophrys*) exposed to a metal mixture (Pb, As, Cd, Cu, Al).

Mitochondrial DNA analyses: One of the most powerful tools of modern molecular population genetics is nucleotide sequence analysis of mtDNA (Bickham *et al*. 2000). The mitochondrial-protein-coding regions are regarded as powerful markers for genetic diversity analysis. One of the most studied mitochondrial genes in genetic diversity analyses is the cytochrome b, the NADH dehydrogenase and mt-cytochrome oxidase I. Also, the highly polymorphic non-coding region of the mtDNA, termed the control region, has been used in genetic diversity analyses due to its role in replication and transcription of mt-DNA. Advantages of the sequence approach include the ability to target different mitochondrial genes, thus, selecting for targets with an appropriate evolutionary rate as well as the higher resolution obtained by revealing the nucleotide sequence. Moreover, and advantage of the PCR-RFLP analysis of the mt-DNA is that homo and heterozygosity values and allele/genotype frequencies can be determined for the genetic loci analyzed (D'Surney *et al*. 2001, Arif and Kahn 2009). Some illustrative examples include: Matson *et al*. (2006) using mtDNA-sequencing, observed that genetic diversity decreased significantly in exposed populations (Hg) of the marsh frog (*Rana ridibunda*). The authors concluded that environmental degradation due to Hg contamination is the most likely cause of the regional reductions of genetic diversity. On the contrary, Eeva *et al*. (2006) using the same biomarker observed increased nucleotide diversity in populations of the Pied flycatcher (*Parus major)* in polluted sites (Cd, Zn, Cu, Pb, Ni, Al, As, Cr) suggesting high mutation rates. These results are in accordance to various field studies which have demonstrated that mutations accumulate more rapidly in more polluted environments (Yauk and Quinn 1996, Clements 2000, Peles *et al*. 2003, Gardestrom *et al*. 2008).

The majority of studies assessing population genetic responses have observed that populations inhabiting more contaminated environments by heavy metals, hold fairly less genetic diversity, as well as, population differentiation, low reproductive success, reduction of the adaptive potential and lower fitness. Also, these responses have been associated with high levels of DNA damage (Blaise *et al.* 2003, Farag *et al.* 2003). Therefore, a potential association between metal contamination and changes in population genetic structure has been suggested.

Finally, the aforementioned studies clearly illustrate that the concept of biomarkers is successful and deserves a place within the theoretical framework of modern ecotoxicology. Bickham *et al*. (2000) suggested that "because population genetic changes are expected to be independent of the mechanisms of toxicity, and sensitive indicators of transgenerational effects, they represent the ultimate biomarker of effect". Because genetic changes, especially the loss of genetic variability, might be permanent (depending on the population size and mutation rates), once variability is lost the population cannot recover to what it was prior to the environmental impact. Also, there is strong evidence suggesting that genetic population diversity may be a useful biomarker of the health of the ecosystem.

Community level biomarkers

At the community level, changes in composition, richness and species diversity may occur as a consequence of exposure to heavily polluted sites, such as superfund sites, where high levels of heavy metals are found. Due to species interactions, such effects cannot be accurately predicted from effects at the population level, as was recognized by Forbes and Forbes (1993), Hopkin (1993), Smith and Cairns (1993), and Lagadic *et al*. (1994).

Studies assessing community level responses to environmental metal stress are mostly conducted in aquatic ecosystems using invertebrate and fish communities. Among the few studies conducted in terrestrial ecosystems, insect communities are the unit of analysis. For example, Theodorakis *et al*. (2000) analyzed the relationship between biomarkers of effect and changes in fish community structure (diversity and percent pollution-tolerant species) exposed to Hg in sediments. They showed a reduction in species diversity at the most contaminated sites, which tended to increase with increasing distance from the pollution source. They concluded that biomarkers of effect are related to community level responses. Also, Clark and Clements (2006) conducted field and stream microcosm experiments to assess community-level responses (composition, species richness) of macro-invertebrates exposed to heavy metals. They established concentration-response relationships between heavy metals and species richness. Similar results were obtained by Pollard and Yuan (2006) with benthic invertebrate communities along a metal pollution gradient. Moreover, Lefcort *et al.* (2010) found that even after a period of 70 years, heavy metals from mining wastes may still be impacting insect abundance and community structure. Speci-

fically, they found that increased Cd and Zn levels were associated with decreased community diversity.

Ecosystem level biomarkers

At this point, it becomes more difficult to relate ecosystem effects exclusively to metal exposure. Therefore, various authors have recommended more rigorous experimental designs coupled with multidisciplinary research, in order to overcome this problem (Medina *et al*. 2007, Hoffmann and Willi 2008). In spite of this, there are concepts that help to understand ecosystems under chemical stress. Here, some of these concepts are addressed.

Risks to the ecosystem and its components are expected to increase as the amount of pollutant entering the system increases, especially when the ecosystem is polluted by heavy metals, because of their bioaccumulation properties and persistence for long periods of time (Hoffmann and Willi 2008). After the ecosystem health is compromised due to heavy metal pollution, there will be a degree of self-compensation in each ecosystem which will tend to preserve its dynamics somewhat. This is known as ecosystem "resistance" (Moriarty 1999), which is analogous to the compensatory responses exhibited by individual organisms exposed to pollutants (Belfiore and Anderson 1998). A resistant ecosystem may show little change in its dynamics if, for instance, loss of one or more species from the ecosystem following pollutant exposure is associated with replacement by alternative species that serve the same role. However, if key species are lost or mostly impaired, such that ecosystem structure and/or function are affected, then the ensuing ecosystem change shows that ecosystem resistance has been overcome (Moriarty 1999). Interestingly, the replacement of sensitive species by more tolerant species without significant changes in ecosystem structure and function could in itself be interpreted as an "early warning" of a pollutant impact if loss of the species can be directly attributed to exposure to a particular chemical. If biomarkers were to be used to measure toxicity in a sentinel species, population decline might well be detected at an even earlier stage. This illustrates an important principle, namely that monitoring changes in populations of sentinel species might provide a valuable insight into the status of the whole ecosystem (Depledge and Fossi 1994).

Many studies have examined the prevalence and distribution of trace and heavy metals in terrestrial food webs (Hunter and Johnson 1982, Beyer *et al*. 1985, Hunter *et al*. 1987). Patterns of uptake and bioaccumulation have been investigated by studying relationships between metal concentrations in soils and plants and in soils and tissues of co-occurring animals (Sharma and Shupe 1977, Otte *et al*. 1990, Shore 1995). These patterns can reveal general trends of exposure, uptake, translocation, and assimilation of metals within organisms. Trophic transfer of metals within the food web may be demonstrated by relating metal levels in dietary components with those assimilated by an animal (Torres and Jhonson 2001). Finally, bioaccumulation of metals in organisms should be included when analyzing ecosystem effects, since some metal effects may only be recognized in a later phase of life, are multi-generation effects or manifest only in higher members of a food-web. Hence, bioaccumulation of chemicals in biota may be a prerequisite for adverse effects on ecosystems (Van der Oost *et al*. 2003).

IN SEARCH FOR NEW BIOMARKERS OF EXPOSURE TO METAL POLLUTION: THE "OMIC" APPROACH

The field of toxicology has recently begun the process of reinventing itself in view of the rapid technological and conceptual change in molecular biology and genomics. The "omic" approach comprises technologies such as genomics, proteomics, metabolomics, transcriptomics, etc. These new ''omics'' disciplines apply high-throughput methodologies which changes in expression of hundreds to thousands of genes (genomics), proteins (proteomics) and metabolites (metabolomics) thatare assessed simultaneously (Snape *et al*. 2004). The combination of high-throughput methodologies such as microarray technology and toxicology led to the development of a new scientific discipline, "toxicogenomics" which is the fusion of toxicology, molecular biology, and bioinformatics (Nuwaysir *et al*. 1999). In particular, toxicogenomics offers not just the possibility of determining which molecular pathways are perturbed by toxic compounds, but also a way of exploiting this information, either for the development of new tests or for the development of new biomarkers (Tugwood *et al*. 2003).

The grand goal of toxicology in the post-genome era is to characterize the entire set of genes and proteins that are affected when humans are exposed to environmental xenobiotics. As a consequence, environmental health scientists can conduct large-scale studies of the effects of toxicants on gene expression at the mRNA and protein levels, while simultaneously monitoring metabolite profiles to gain insight into the activity

state of all relevant genes and gene-products. A direct comparison of expression values obtained for a control versus an altered condition reveals a set of biomarkers indicative of that altered state. This exposure ''signature'' can then be used as a tool for classifying chemical exposures and predicting mode of action (Hamadeh *et al*. 2002, Olden 2006). Specifically for metal exposure assessment, very recently, "metallomics" has emerged as a new sub-discipline of toxicogenomics, which investigates the interrelationships of metal-induced proteome and metabolome changes. In this regard, searches for genes encoding metal-responsive proteins could be interesting targets for reporter genes fusions in biomarker establishing (Haferburg and Kothe 2010).

Furthermore, the integration of the "omic" approach with ecotoxicology, led to the term ''ecotoxicogenomics'' which includes gene-protein level responses that directly affect population and community dynamics via developmental or reproductive perturbations (Snape *et al*. 2004). Effort towards linking these molecular signatures with alterations in the genetic pool of the affected populations is envisaged. Only then, we will be able to say that "omic" technologies not only help to provide novel biomarkers but also a close look to the continuum of toxic responses from molecules to ecosystems. However, traditional biomarkers targeted for these affected systems should be used to validate the toxic mechanisms of the contaminant. Additionally, one must consider that an expression profile is merely a "snapshot" of a highly dynamic system, and temporal changes in gene and protein expression should be anticipated.

To date, most of the work using DNA microarrays have focused on genetically well characterized organisms, including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*. However, a major obstacle to the application of microarrays in ecotoxicology, is the lack of genomic or cDNA sequence data for sentinel species and non-model organisms (Snape *et al*. 2004, Mehinto *et al*. 2012). In spite of this, gene expression arrays are being developed for a number of non-model organisms; in a variety of fish species (Gracey *et al*. 2001, Jeffries *et al*. 2012), frogs (Altmann *et al*. 2001, Blackshear *et al*. 2001) and birds (Morgan *et al*. 2001, Neiman *et al*. 2001).

One of the best examples when trying to search for new biomarkers of exposure to environmental pollution, was the study conducted by Venier *et al.* (2006) who uncovered over 40 novel biomarkers whose expression levels were regulated similarly in the laboratory and field exposures. Other examples that have illustrated the usefulness of these technologies and have discovered new biomarkers for environmental metal exposures are Wang and Fowler (2008), Ki *et al*. (2009), and Menzel *et al.* (2009).

One of the major disadvantages of gene expression microarrays, is that the analysis of data is complex. There has been some consensus about analysis approaches (Allison *et al*. 2006) but lack of standardization in approaches has introduced difficulties when comparing results between laboratories (Quackenbush 2006).

In spite of these limitations, these novel technologies offer added value compared with classical testing with whole organisms because they provide information concerning the molecular basis of exposure and act as "early warning" signs, enabling a more robust risk assessment than has ever been achieved previously. These new methods might also help to provide data that could reduce much of the uncertainty in extrapolating from laboratory animals to human exposures. Moreover from an ecotoxicological perspective, it is expected that these new methods will provide a better understanding on the application of uncertainty factors that are used to extrapolate data from laboratory to field and from sentinel species to the whole-ecosystem level. More studies are needed to further define the potential applications and limitations of genomics in biomarker research.

CONCLUSIONS AND FINAL REMARKS

From the examples given in these review, it is clear that environmental metal exposure can elicit a plethora of biological effects, ranging from alterations in molecules, compromising individual health and putting ecosystem integrity at risk. Therefore, in each level of biological organization a set of biomarkers can be measured in order to integrate an holistic perspective of complex environmental exposures. Biomarkers at the cellular or sub-cellular levels are adequate, useful and in some cases, well established. However, the use of biomarkers beyond the individual level has not always allowed for cause-effect relationships, since more confounding factors are present, few specific biomarkers are available and often measuring biological responses in field situations becomes difficult.

A major limitation of biomarker use is that a variety of responses have been identified in exposed organisms, making difficult to link environmental exposure to specific chemical entities and subsequent biological effects. In this case, the use of a

multi-biomarker approach, in a range of species using sentinel organisms, becomes necessary to resolve or at least have a closer insight into complex environmental problems. Also, there is a recognized need for biomarker research to move toward a more holistic approach, a proposal that is in harmony with the power of genomics as a tool for understanding toxicant impacts in a diversity of species.

Overall, approaches that integrate responses across levels of organization are especially valuable because they help to understand the mechanistic linkages between the biomarkers responses and the ecologically relevant responses. Therefore, choosing the appropriate biomarker must be based on the biological level of organization in question.

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