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Research

USE OF MOLECULAR BIOMARKERS IN STUDIES OF AQUATIC ENVIRONMENTAL IMPACT

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ABSTRACT

Molecular biomarkers refer to specific genes and/or its products present in ecosystems which have been exposed to physic or chemical alterations as a result of anthropogenic activities. These molecules are useful to estimate both change and spread of the damage level. The studies of DNA specific sequences, gene expression and proteins production allow to understand the consequences of the presence of specific pollutants or its mixtures in different ecosystems, but mainly in aquatic environments. To date, different gene targets have been used to understand the consequences derived by the introduction of specific pollutants into the environment and serve as excellent tools for environmental toxicology studies. In this review, some of the most commonly reported molecular biomarkers used to monitor the environmental impact in aquatic ecosystems and the pertinent considerations when they are used in environmental risk assessment studies are described.

INTRODUCTION

The measurement of environmental risk derived of the growing socio-economic activities demand the fast development of effective and reliable tools, able to estimate the effects of different pollutants in the ecosystems. The measurement of different biochemical, histological, morphological and behavioral parameters has been commonly used to monitor the impact in ecosystems (Peakall, 1994; Peakall and Walker, 1994; Vincze et al., 2015). However, these approximations could just be final observations of a sub-lethal chain of events, in which case, would allow to better explain the effects derived from the introduction of the pollutant. Thanks to the advances in molecular biology now, there are available tools that allow the identification of molecular biomarkers, such as the translation of specific proteins, quantification of mRNA from specific genes related with the presence of pollutants and the identification and quantification of changes in the structure or sequence of DNA, among others. All of these strategies are named molecular biomarkers.

Molecular biomarkers allow to identify biological responses at different levels, e.g. the whole organism (animal, plant, bacteria, etc.) or its compartments (tissues, cells, sub-cellular organelles) once the pollutants or chemical stressors are present in the environment. This approximation allows to know sub-lethal metabolic and physiological reactions in different types of organisms (Muñoz et al., 2015; Giuliani and Regoli, 2015; Milan et al., 2015). Additionally, molecular biomarkers can permit the integration of typical measures of physical-chemical pullulans and biological responses (Capela et al., 2016).

For the development and use of a molecular biomarker, it is necessary to know variables that could affect their expression and, in consequence, lead to confuse interpretations. These variables are: i) physical parameters such as the geography, weather conditions, mineral compositions of water and sediments and anthropogenic impact; ii) the pollutant or the stressor itself, which generally is a mixture of organic molecules or metals with different solubility and different ability to be taken from the ecosystem; iii) target species, that could be either endemic or introduced and iv) molecular biomarkers, the specific molecule or response used to measure the extent of the impact. In this review,

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some of the most commonly reported molecular biomarkers used to monitor the environmental impact in aquatic ecosystems and the pertinent considerations when they are used in environmental risk assessment studies are described.

PHYSICAL PARAMETERS

The study of molecular biomarkers allows the identification of side effects derived from pollutant contamination in water and soil (Asensio et al., 2013; Cansaran-Duman et al., 2011; Celander, 2011; Oztetik, 2015). However, aquatic environments have gained interest because of their complexity (Colin et al., 2016). Aquatic ecosystems are a wide range of different systems as lagoons, rivers, underground aquifers, estuaries, sea and oceans. Each system is influenced by the weather, water flow and the level of anthropogenic or industrial stress they receive, as well as the interactions between pollutant and target species (Costa et al., 2012).

Seasonal variations induce changes in temperature and pH of the ecosystems allowing the spread of microorganism like cyanobacteria and algal blooms, the proliferation of microorganism which in turn decreases the oxygen availability or the production of toxic substances that could induce the expression of proteins related with oxidative damage, such as glutathione-S-transferase (Cheng, 2012; Falfushynska et al., 2010). Climate changes could increase the bioavailability of Polycyclic Aromatic Hydrocarbons (PAHs), metals such as cadmium (Cd) and lead (Pb) (Costa et al., 2012), and increase the mRNA expression in fish exposed to Cadmium chloride (Van Cleef-Toedt et al., 2001). The physicochemical composition of sediments and soil near aquatic environments 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 (Boshoff, 2014).

STRESSORS

The impact derived from anthropogenic activities has changed the distribution of many molecules and compounds in our planet. In the same way, the oil industry has introduced organic substances into the superficial ecosystems, and many other industries have isolated, purified and concentrated molecules and ions which under natural conditions would be present at low levels. In parallel, the synthesis of new compounds for agroindustrial and pharmacological purposes has increased the number of pollutants released into ecosystems (Bi et al., 2015; De Castro-Català et al., 2015). There is no unique classification for environmental pollutants, but overall, they can be grouped according to their structure, source or the final effects caused in target species. In this review they are classified into three major groups: i) toxic organic compounds such as PAHs, endocrine disrupting compounds (EDCs), perfluorinated compounds (PFCs) and alkylphenols (AP), ii) metals and iii) emerging pollutants such as pesticides and pharmaceutical active compounds-PhACs (De Castro-Català et al., 2015). Organic compounds are mostly hydrophobic and recalcitrant (Boitsov et al., 2007) and they tend to stay for a long term in the ecosystems. This fact allows chronic interactions of pollutants with aquatic species and facilitates their accumulation through the skin and other tissues (Jhonston, 2015). For example, PAH recalcitrance lead to the expression of cytochrome P4501A in mussels, even months after the first exposure episode (Sureda et al., 2011). At the same time PAHs could be metabolically activated into reactive compounds, forming adducts. Pesticides and PhACs present similar metabolic mechanisms as PAHs, and are finally biotransformed by hepatic cells (De Castro-Català et al., 2015; Thompson et al., 2010). Metals can be recognized and transported by metallothioneins, which increase their concentration in blood in gills, gut and soft organs (Roesijadi et al., 2009). However, metal accumulation in soft organs is heterogeneous and can be confined to subcellular compartments that influence the appearance and distribution of Metallothioneins (MT) (Le et al., 2016).

TARGET SPECIES

Different studies have been recently conducted in water bodies using fish or mussels aiming to establishing close relationships between the presence of a molecular biomarker and pollutant levels. The selection of the target species is a key aspect in biomarker studies, because it can determine the usefulness of the molecular biomarker selected but it can also induce mistakes when the final correlations are made. Most studies use motionless species (mussels) to avoid the bias produced by the migrations and the changes from polluted environments to pollutant-free zones (Auslander et al., 2008; Costa et al., 2012; Dallas and Jha, 2015; Regoli et al., 2011). Thus it becomes necessary to know the distribution, life cycle and ecological traits of the native species present in ecosystems, since this conditions could increase the ecological relevance of biomarkers. Species sharing the same ecosystem are probably not affected in a similar extent by pollutants, as observed in a study on *Dicentrarchus*

labrax and *Liza aurata*, showing an overexpression of CYP1A1 gene and more DNA damage in L. *aurata* more than in D. *labrax*, even in non-polluted areas (Nogueira et al., 2010). On the other hand, it has been observed in mussels that food sources and biological indices as absorption efficiency and respiration rate can also modulate the accumulation of metals as Pb, cooper (Cu) and arsenic (As) in mussels, changing the level of expression of biomarkers (González-Fernández et al., 2015).

The life cycle of the target species must be fully known, as younger animals tend to be more frequent in upriver areas and they are present only in some specific months (Capela et al., 2016). For example, the expression of multixenobiotic resistance proteins (MXR) (e.g. ABC transporters) in Nile tilapia depends on their stage of development, showing that the interactions between xenobiotic and target species is different at every stage of the life cycle, and consequently, each stage has specifically genes to be measured. The same has been observed in zebrafish, which has 47 different cytochrome P450 genes (in contrast with 16 in human) and its expression depends on the life cycle stage (Costa et al., 2012). Usually, endemic species are preferred over foreign species because the latter may need a long time to adapt to a new ecosystem, and on the other hand, the responses of the introduced species to pollutant accumulation or DNA damage could be absent (Nigro et al., 2006). This fact led to using model species, like the zebra fish. Using model species have some advantages, since some of them are extensively studied to have their complete genome sequenced. Recently, a study using commercial fish *Carassius auratus* showed this species could be used to quantify the expression of early biomarkers such as antioxidant defense systems. Additionally, the advantages of using model species allow making comparisons among different polluted areas, as well as the use of genetically modified species in lab that facilitates the measurement of the impact from different areas (Mutwakil et al., 1997).

MOLECULAR BIOMARKERS

Different strategies to measure the presence of pollutant in exposed areas, such as the direct measurement of the pollutant in tissues, the identification of metabolites derived from the pollutant by biotransformation inside the target species, cellular and histological alterations and molecular changes have been described in experimental studies. This review is focused on molecular biomarkers (DNA, RNA and protein) because they can identify the presence the pollutants in very low concentrations inducing different types of metabolic and sub-lethal effects in target species before they cause clearly noticeable damages as a consequence of their accumulation. A sub-lethal effect could be an activation, inactivation, over expression or interference effect of different metabolic routes in response to the presence of a xenobiotic compound. To measure these changes, molecular tools such as qPCR, RT-qPCR, DNA, RNA microchips and 2D protein electrophoresis have been developed to quantify specific changes in gene expression during the stress episode. These tools allow the identification of changes in the expression of specific genes related with diseases or the interference with metabolic routes resulting in physiologic alterations. Additionally, molecular tools can provide information about the genomics, transcriptomics, proteomic and metabolomics of target species. In other cases, these tools can be useful when the global information about native species in not available, or direct the selection of new target genes to use as early alerts (Asensio et al., 2013; Miao et al., 2015). To date, there are numerous target genes to identify the responses toward different pollutants. These targets genes or its products are grouped in five categories: i) Biotransformation enzymes, ii) Oxidative and cellular stress-related proteins, iii) Heavy metal-associated proteins or metallothioneins, iv) Endocrine system-associated proteins and v) Cell death-related proteins. Some of these groups will be discussed.

BIOTRANSFORMATION ENZYMES

Biotransformation enzymes are part of a large family of proteins responsible for the metabolism of xenobiotics, such as PAHs and polychlorinated biphenyls (PCBs). As most of these are highly hydrophobic compounds in nature are thus easily incorporated into the organisms by ingestion, inhalation or dermal absorption. Biotransformation enzymes participate in the solubilization and removal of these pollutants to avoid its bioaccumulation and entering the trophic chain (Goodale et al., 2015). The most studied genes are those encoding monooxygenases of the cytochrome P4501A complex, also known as CYP1A1. Their physiological role is the metabolism of xenobiotics, catalyzing the oxidative biotransformation, reduction and hydrolysis of substrates such as PAHs and PCBs by the activation of aryl hydrocarbon receptor (AHR). This allows transforming these nonpolar compounds into more water-soluble substances that may be excreted by the organism (Table 1).

As a significant feature, CYP1A1 is inducible by its

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Table 1. Molecular biomarkers used in environmental risk assessment studies.

Group	Biomarker name	Symbol	Associations/Biological processes	References
Biotransformation enzymes	Eukaryotic cytochrome P450	CYP1A	Exposure/biotransformation of aromatic and planar organochlorine compounds	Costa, 2012; Liu et al., 2015 Liu et al., 2014
	Ethoxyresorufin-o-deethylase	EROD		
	Glutathione S-transferase	GST	Exposure/biotransformation of xenobiotics such as PAHs, PCBs and PCDDs.	Cheng et al., 2012
	Glutathione	GSH		
Oxidative and cellular stress proteins	Superoxide dismutase	SOD	Over expression in some tissues such as gills, but mainly in liver after the detoxification process.	Chen, 2012; Loro, 2012, Giarratano et al., 2014
	Catalase	CAT		
	Glutathione peroxidase	GPx		
	Glutathione reductase	GR		
	Peroxidase	POD		
	Lactate dehydrogenase	LDH	Cellular lysis and tissue damage.	Clark, 2010
	Heat shock protein 70	HSP70	Produced in response to chemical or physical stressors.	Clark, 2010; Lehnert et al., 2014
Metal-binding cysteine-rich proteins	Metallothioneins	MTs	Compensatory mechanism during exposure to heavy metals (Cd, Fe, Hg, Zn, As)	Roesijadi et al., 2009; Ghedira et al., 2016
Endocrine system proteins	Vitellogenin	VTG	Alteration or disruption of hormonal axis by mimicry	Dos Anjos et al., 2011; Costa et al., 2012
	Vitelline envelope proteins	VEPs		
	Cytochrome P450 aromatase	CYP19A		
	Acyl Coenzyme A Oxidase	ACOX1	Produced after exposure to oil hydrocarbons	Ruiz et al., 2012
Programed cell death proteins	Direct IAP-binding mitochondrial protein	DIABLO (SMAC)	Found in liver after exposure to PCBs.	Zacchino et al., 2012
	Caspases	CASP2-10	Initiators of programmed cell death when the stressor overcome rescue mechanisms in the cell.	Zacchino et al., 2012
DNA integrity markers	Micronuclei		It can overcome 8.8 times its expression after oil spills in mussels	Baršienė et al., 2012
	DNA adducts		Exposure of DNA to intercalating aromatic planar compounds.	Santos et al., 2006
	DNA fragmentation			Costa et al., 2012

own substrate, in this case the xenobiotic molecule; thus, their activity is only evident after the organism is in contact with these compounds. For this reason, the quantification of mRNA, protein concentration, and the resulting enzyme activity of CYP1A1 are used as biomarkers to assess the exposure to these pollutants. In aquatic ecosystems, CYP1A is widely monitored in fish and mollusks (Bonnineau, 2012), which described a strong association of the expression and the presence of aromatic hydrocarbons. In mussels exposed to oil spills, CYP1A levels begin to increase one month after exposure and remain detectable after six months (Sureda et al., 2011). In fish, there is consistency among the results at field and laboratory scale and they remain stable through different seasons (Costa et al., 2012). The expression of these genes has been studied in bacterial populations in order to identify its association with the presence of pollutants, as demonstrated with Geobacter uraniireducens, which overexpressed cytochrome associated-genes when in presence of uranium (VI) (Holmes et al., 2009). This highlights the usefulness of the quantification of these genes to identify contamination episodes with these pollutants.

HEAVY METAL-ASSOCIATED PROTEINS

Heavy metal-associated proteins are high molecular weight proteins rich in cysteine and sulfhydryl groups, which interact with metal ions and allow

their transportation through the circulatory system. Their expression has been studied in response to oil spills in water bodies. Along with CYP1A, their use has been proposed for evaluating the effectiveness of detoxification mechanisms in oil spill sites (Sureda et al., 2011). The expression of heavy metal-associated proteins is commonly assessed in gills and liver of the aquatic affected species. One advantage of this molecular biomarker is that genes coding for these protein is highly conserved within the species used as sentinel organisms, such as mollusks and fishes. Overexpression of metalloproteins´ mRNA has been observed in fish and shellfish exposed to high concentrations of zinc (Zn), aluminum (Al), Cu, Pb and Cd (Giarratano et al., 2016).

RESPONSE TO ESTROGENIC CHEMICALS

Pollutants such as PAHs have the ability to interfere in the endocrine system of several organisms allowing the expression of proteins such as vitellogenin (vt), a glycolipoprotein acting as a precursor of bud formation in most oviparous species. Studies in river fish have shown that PAH-like compounds such as 3-methylcytosine (3MC) and β-naphthoflavone (βNF) decrease vitellogenin synthesis by binding to AhR receptor, triggering an anti-estrogenic response (Navas and Segner, 2000; Fort et al, 2015).

In female organisms the alteration of vt serum levels is useful as a marker of reproductive toxicity, as chemicals inhibiting the conversion of testosterone to estradiol by the interaction with the enzyme aromatase (CYP19) has been associated with reduced levels of estradiol, followed by a decrease in vt levels. Miller et al. demonstrated the relationship between the concentration of vt and fecundity of female fish, and how this may affect the decrease of population number. The induction of vt in young male fish is useful to estimate the exposure to estrogenic chemicals and act as an early warning of exposure to chemicals agonists to the AR in aquatic environments (Fort et al., 2015).

DNA INTEGRITY

DNA integrity tests are designed to determine the alteration of the genome at structural level, as evidenced by morphological changes in its distribution at intracellular level (micronucleus assay), molecular structural changes (presence of adducts) or loss of DNA integrity (comet assay). DNA damage is one of the most important alterations since they may produce neoplastic or pre-neoplastic diseases, being able also to affect germinal cells and therefore affect an entire population. Exposure studies on fish show that pollutants such as PAHs may induce consistent changes in the genome after 28 days of exposure (Costa et al., 2012). This could be due to the ability of PAHs and their intermediates to covalently bind to DNA, decreasing the DNA repairing ability of cells. In addition, some of these act as photoactive molecules leading to the formation of oxygen free radicals that contribute to the formation of breaks in DNA molecules. Nuclear or mitochondrial DNA injuries can be measured by using Large amplicon quantitative PCR (LA-QPCR), which allows quantifying the extent of the damage (Jung et al., 2009).

PROTEOMICS

The description and deposit of DNA and mRNA sequences, from multiple fish or mussel species, into publicly available databases has allowed the quantitation of mRNA expression from specific genes, but it could also limit the responses that can be obtained from biological samples. The analysis of protein expression allows the identification of proteins, which either had not been previously considered as biomarkers, or simply had not been studied in the presence of contaminants. Proteomic studies from mussels after metal exposition have showed an overexpression of new proteins not considered as biomarkers. These enzymes were mainly related with carbohydrate metabolism, such as triose phosphate isomerase, G-protein beta subunit, phosphoenolpyruvate carboxykinase as well as others related with glycolysis and Krebs cycle in humans, and they showed to respond in a dose-dependent manner after expositions to pharmaceutical compounds (Pedriali et al., 2013; Thompson et al., 2012). In other studies, proteomics has revealed new unidentified proteins on mussels exposed to PhACs (Schmidt et al., 2014).

INTERPRETATION OF THE INFORMATION DERIVED FROM MOLECULAR BIOMARKERS

The use of molecular biomarkers as indicators of ecosystem pollution should be undertaken with caution. Some studies have shown there are situations that can make difficult the interpretation of these results:

• The expression of a biomarker gene may be triggered by a specific concentration of pollutant, so that at higher or lower concentrations they may not be detectable.

• Certain biomarkers are subjected to the adaptation effect of the individual, being undetectable after long periods of exposure.

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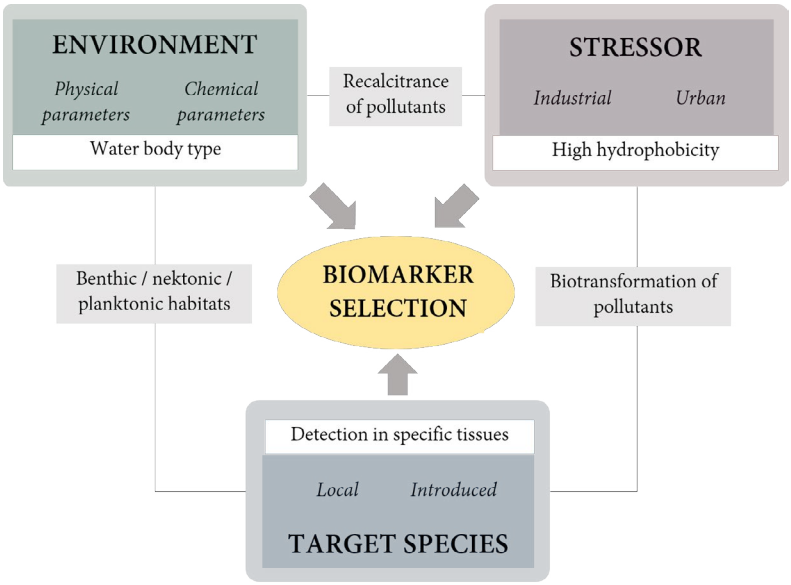

Fig 1. Interactions defining the selection of a biomarker. An adequate selection of molecular biomarkers needs the identification of pollutants in the aquatic environment, as well as the environmental factors, target species and their interactions. Those interactions are influenced by biotransformation of the stressors, their degree of recalcitrance, as well as the habitat of target species.

• Some environmental conditions, such as the season, modify the expression of biomarkers as they alter the availability of contaminants in the environment (Barhouimi, 2012).

• Most biomarkers are regressive and return to baseline when exposure stops, which does not allow the identification of exposures before the time of the study.

• Tests based on the detection of RNA expression, although more rapid and sensitive compared with other biomarkers (such as pathological findings or direct evidence of bioaccumulation) should be developed taking into account a good selection of reference genes (Lacroix et al., 2014).

• Biomarkers expression may be influenced by factors such as food availability or rainy/dry seasons, masking pollutant effects. This situation can be solved with the use of reference areas in which the behavior through different seasons is known (Benali et al., 2015).

Despite the above, there have been approaches utilizing biomarkers to estimate ecosystems damage levels. An example of this has been reported by Benali et al. (2015), using enzymatic biomarkers along with morphological parameters to classify the toxic potential of coastal sites.

CONCLUSIONS

The analysis of molecular biomarkers represents a highly sensitive method for the monitoring of endemic species exposed to stressor molecules. The way in which the measurement is made will make possible to identify the extent of the impact. The use and adequate selection of target genes and sentinel species could offer the relevant information about the grade of impact of individual specimens and its later correlations and use to scales in order to measure the environmental risk assessment. An adequate selection of molecular biomarkers needs the identification of pollutants in aquatic environment, as well as the abiotic factors, stressors, and their interactions (Figure 1). Thus, the measurement of biomarkers expression could be integrated with other analysis useful for the risk assessment of hazardous substances (Lee et al., 2015). The description of their uses and limitations allows the use of molecular biomarkers as a line of evidence for studies of environmental risk assessment. Results from studies using molecular biomarkers are now being used by international groups such as OSPAR and HELCOM, for the monitoring of protected areas like inland surface waters, transitional waters, coastal waters and ground waters.

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