

Ecotoxicol. Environ. Contam., v. 11, n. 1, 2016, 53-61 doi: 10.5132/eec.2016.01.08



Confounding factors in biomonitoring using fish

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(Received February 20, 2014; Accept September 14, 2016)

Abstract

Pollution of water ecosystems is considered one of the most serious problems in the current days. In order to predict the effects of pollutants present in the water before overt disease and/or death in aquatic biota, bioindicators, as fish, and biomarkers have been widely used in biomonitoring studies. Notwithstanding, biomarkers responses might be modulated by non-pollutant factors and not only by environmental contaminants. As these premises are not always observed, this review attempts to explore confounding factors related to the modulation of the biomarker responses applied in biomonitoring studies. Among others, gender, temperature and feeding habitat are some factors which affect biomarker responses. However, the use of this tool is still important to assess the health of aquatic ecosystems. Thus, it is strongly recommended to use different biomarkers and, whenever possible, to analyze water and sediment physicochemical parameters.

Keywords: biomarkers; biomonitoring; fish; pollution; environment; environmental quality; water resources.

INTRODUCTION

Efforts have been ongoing in many countries to develop integrated ecosystem-based assessments using physicochemical properties, biological abundance and diversity, and/or chemical characterization to define the ecological quality of aquatic environments (Hellou, 2011). Pollution, loss of biodiversity and habitat destruction are probably the main environmental threats for aquatic ecosystems (Linde-Arias et al., 2008a). Increasing number and amount of industrial, agricultural and commercial chemicals discharged into the aquatic environment have led to various deleterious effects on the aquatic organisms (McGlashan & Hughies, 2001; Procópio et al., 2014). Bioconcentrations of highly persistent pollutants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides and toxic metals in species of marine organisms, and/or biomagnifications along the trophic chain, are considered one of the mayor threats to human and ecosystem health (Tomasello et al., 2012). In an attempt to monitor adverse effects caused by pollution, bioindicator organisms and biomarkers have been widely used as tools in environmental diagnose.

Fish are widely used as biondicator organisms and they represent the most feasible organisms for pollution monitoring in aquatic systems (Linde-Arias *et al.*, 2008b). Fish can be found virtually everywhere in the aquatic environment and they play a major ecological role in aquatic food-webs because of their function as carrier of energy from lower to higher trophic levels (van der Oost *et al.*, 2003).

Aquatic organisms are exposed to a wide variety of environmental contaminants. Thus, a set of biomarkers is frequently employed to assess the possible biological impacts and environmental quality. Biomarkers are increasingly worldwide-recognized tools for the assessment of pollution impacts in the aquatic environment, and some of them are already incorporated in environmental monitoring programs (Viarengo *et al.*, 2007). Biomarkers can be characterized as functional measures of exposure to stressors, which are usually expressed at the subcellular level of biologic organization (Adams *et al.*, 2001). Subcellular responses to environmental stressors occur before other disturbances, such as disease, mortality, or

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population changes, and thus may offer early warnings of pollution impacts (Depledge & Fossi, 1994).

The application of biomarkers for environmental monitoring requires an extensive knowledge of natural variability of biomarker responses. A number of factors such as sex, age, season, spawning period, variability of feeding habitats, pollutant exposure and phylogenetical differences in regulatory mechanisms may influence the uptake, retention and bioaccumulation of trace contaminants in fish tissues (Nesto *et al.*, 2007), consequently affecting biomarker responses. The main confounding factors which might interfere in biomarker responses discussed in this review are listed in table 1. This poses careful interpretation of biomarker responses *per se*, since increased or decreased responses might be due to non-pollutant factors and be unrelated to exposure to chemical pollution. Nevertheless, the aim of this study is to gather data

regarding confounding factors in the biomarkers used in fish in biomonitoring studies.

Tissue differences of pollutants accumulation (metals and PAHs)

Bioaccumulation of pollutants is usually employed combined with water physicochemical analysis or with other biomarkers. This analysis has been useful in order to correlate concentration of pollutants in the water/sediment and in fish tissues; and to assess chronic exposure to pollutants. The body content of a trace metal in a given organism results from the net balance between the processes of metal uptake and metal elimination (Birungi *et al.*, 2007; Paulino *et al.*, 2014). The rate of bioaccumulation of pollutants in fish might be higher or lower depending on the analyzed tissue. In the case of the grass goby (*Tridentiger bifasciatus*), a near-botton feeder,

Table 1: Main confounding factors which might affect biomarker responses to pollutants

Biomarker	Likely affected by	Reference
Acetylcholinesterase (AChE) and cholinesterase (ChE) activity	Presence of arsenic, copper and mercury, detergents, seasonal variation and temperature	Lagadic et al., 1994; Guilhermino et al., 1998; Beauvais et al., 2002; Phillips et al., 2002; Napierska & Podolska, 2005; Pfeifer et al., 2005; Kopecka & Pempkowiak, 2008; Durieux et al., 2011; Jolly et al., 2012; Botté et al., 2013
Bioaccumulation of metals in tissues	Tissue itself, feeding habitat, presence of ions of calcium (for manganese and copper accumulation) and copper (for zinc accumulation)	Roch <i>et al.</i> , 1985; Seymore <i>et al.</i> , 1995; Dallas & Day, 1995; Croteau <i>et al.</i> , 2005; Birungi <i>et al.</i> , 2007
Bioaccumulation of polycyclic aromatic hydrocarbons (PAHs)	Nutritional status and metabolic rate	Collier & Varanasi, 1991; Brumley <i>et al.</i> , 1998
Condition factor	Disease, nutritional status and seasonal variation	Mayer et al., 1992
Gonado-somatic index	Nutritional status and seasonal variation	Nesto et al., 2007
Ethoxyresorufin O-deethylase (EROD)	Age, estradiol, gender, presence of copper and cadmium, seasonal variation and temperature	Förlin & Andersson, 1984; Andersson & Förlin, 1992; Roméo <i>et al.</i> , 1994; Whyte <i>et al.</i> , 2000; van der Oost <i>et al.</i> , 2003; Kashiwada <i>et al.</i> , 2007; Guilherme <i>et al.</i> , 2008; Vieira <i>et al.</i> , 2009; Wunderlich <i>et al.</i> , 2015
Glutathione peroxidase (GPx)	Seasonal variation	Sanchez <i>et al.</i> , 2007; Jolly <i>et al.</i> , 2012
Glutathione S-transferase (GST)	Gender, physiological factors, seasonal variation and spawning period.	Elia <i>et al.</i> , 2003; Vieira <i>et al.</i> , 2009; Jolly <i>et al.</i> , 2012
Heat Shock Protein 70 (HSP70)	Nutritional status, temperature, water physicochemical parameters	Stegeman <i>et al.</i> , 1992; Di Giulio <i>et al.</i> , 1995; van der Oost <i>et al.</i> , 2003
Lipid peroxidation (LPO)	Feeding habitat	Radi et al., 1985
Metallothioneins (MTs)	Age, gender, breeding period, presence of organic contaminants, seasonal variation, temperature and tissue	Gerpe <i>et al.</i> , 2000; van der Oost <i>et al.</i> , 2003; Sokolova & Lanning, 2008; Rhee <i>et al.</i> , 2009; Fonseca <i>et al.</i> , 2011; Shariati & Shariati, 2011; Carvalho <i>et al.</i> , 2012; Siscar <i>et al.</i> , 2014
Superoxide dismutase (SOD) and catalase (CAT)	Age, feeding habitat and nutritional status	Radi <i>et al.</i> , 1985; Mourente <i>et al.</i> , 2002; Rueda-Jasso <i>et al.</i> , 2004; Ameur <i>et al.</i> , 2012; Hook <i>et al.</i> , 2014

sediments may also play an important role as a source of contaminated food (Croteau *et al.*, 2005). This correlation between metals in fish tissues and sediment has been found in some studies (Nesto *et al.*, 2007; Weber *et al.*, 2013).

Gills are central in the uptake of dissolved substances from water representing the prime target for toxic action of waterborne metals (Soto *et al.*, 2003). Therefore, concentrations of metals might be higher in this organ. This finding has been reported in a study which analyzed metals in gills, liver and muscle. Metal concentration was higher in the gills and lower in the muscle (Birungi *et al.*, 2007). On the other hand, considering the liver is the main organ responsible for biodegradation, Paulino *et al.* (2014) observed higher concentrations of contaminants in the liver of *Astyanax fasciatus* and *Pimelodus maculates* when compared to the gills.

Although the rate of accumulation might be influenced by the feeding habitat of the fish species and the analyzed tissue, it might also be influenced by the presence of certain ions. Calcium concentrations in the water might play a role in copper and manganese accumulation. Ions of calcium and copper compete for binding sites in gills and other organs (Lloyd, 1992), so when analyzing copper in tissue of fish from water with high concentration of calcium, the levels of this metal might be decreased (Birungi et al., 2007). Also, in alkaline conditions, copper precipitates as the carbonate and it is non toxic (Dallas & Day, 1993), so pH might also have a role, making copper biologically unavailable (Birungi et al., 2007). Concerning manganese, a competition between manganese and calcium ions also occurs, thus decreasing the bioavailability of manganese to fish (Seymore et al., 1995; Birungi et al., 2007).

Zinc has the ability to bind to metallothioneins in the liver, but since copper has a greater affinity for proteins, zinc concentration might be low due to the presence of copper (Roch *et al.*, 1985). pH might also affect concentration of soluble zinc, where high concentrations of this metal are found in well oxidized conditions (pH 5 to 6.5), whereas low concentrations might be observed in redox conditions (pH 8) (Gambrell *et al.*, 1991).

Concerning PAH accumulation, some studies have indicated that the PAH metabolite levels in bile might be highly influenced by the feeding status of the fish (Collier & Varanasi, 1991; Brumley *et al.*, 1998). An alternative used to reduce variations in PAH metabolite bile levels due to feeding status, is using procedures in which the metabolite concentrations are related to the biliary pigment contents or levels of total protein. In addition, PAH concentrations might be low due to the high metabolic rate in fish (Nesto *et al.*, 2007).

Metallothioneins (MTs)

The correlation between MTs expression level and degree of pollution is particularly evident, confirming that their expression levels are influenced by various metals (Berthet *et*

al., 2005; Sinaie et al., 2010). Consequently, this biomarker has been used in several studies in order to evaluate metal exposure (Nesto et al., 2007; Linde-Arias et al., 2008b; Fonseca et al., 2011; Jolly et al., 2012; Tomasello et al., 2012; Fasulo et al., 2013; Falfushynska et al., 2014). Nevertheless, confounding factors such as seasonal variation, age, reproductive status, water temperature, tissue and individual variation, could contribute to MTs expression modulation, interacting with each other or acting all together (Rhee et al., 2009; Shariati & Shariati, 2011).

Among these factors, temperature is well known to modulate the aquatics specimens' sensitivity to metals by affecting physiological tolerance, energy demand and oxygen supply and/or mitochondrial biogenesis (Sokolova & Lanning, 2008). Siscar *et al.* (2014) have observed a significant effect of temperature on MT content in the liver of *Solea senegalensis*.

Moreover, it has been hypothesized that the presence of organic contaminants reduces MTs synthesis by increasing the demand for cysteine residues of glutathione (GSH), a very important cellular antioxidant molecule (van der Oost *et al.*, 2003). Low MT levels have been observed in sites with low metal contamination, but also in polluted sites characterized by metals contamination, petroleum/crude hydrocarbon inputs and combustion PAH sources (Fonseca *et al.*, 2011). Thus, it demonstrates the ability of organic contaminants in reducing MT levels even in metal contaminated areas.

Furthermore, there is an existing variation in MTs expression among tissues. A higher expression has been found in muscle than in the liver. This could be due to the lower metal-binding capacity (metal uptake and/or accumulation) of the liver in respect to the muscle, a distorted mechanism of membrane transport and an altered mitochondrial biogenesis with consequent reduced energy power or oxygen supply, all induced by metal intoxication (van der Oost *et al.*, 2003). Comparing MT expression in liver and gills, increased levels of MT have been found in gills and it could be attributed to the fact that this organ is the first route of metal uptake (Carvalho *et al.*, 2012). The gills are more susceptible to the immediate (acute) effects of exposure to waterborne contaminants and livers are subjected to the more prolonged (chronic) effects of accumulated contaminants (Cerqueira & Fernandes 2002).

Hormones have also been shown to influence MT levels. In female fish, 17β -estradiol indirectly inhibits MT by altering the zinc utilization in the liver during vitellogenesis (Gerpe *et al.*, 2000).

Biotransformation enzymes

The first stage of detoxification of xenobiotics involves enzymatic transformation of a chemically modifying lipid soluble toxin into water-soluble toxin. Most of the transformation reactions in this phase involve a broad family of enzymes, cytochrome P450s (Lardone *et al.*, 2010). The ethoxyresorufin O-deethylase (EROD) activity is considered a sensitive catalytic probe for determining the induction of the cytochrome P450 system in fish by micro-organic pollutants,

particularly PCBs (van der Oost et al., 2003). Its activity is also considered a specific biomarker of PAH-like compounds exposure (Fonseca et al., 2011). EROD activity induction may be inhibited by estradiol, a hormone produced by mature females (Förlin & Andersson, 1984). Therefore, higher levels of EROD response can be explained by the negative effect of endogenous estradiol on cytochrome P-450 catalytic activity (Arukwe & Gokoyr, 1997). Consequently, this effect is more pronounced during breeding periods. This effect has been reported by some authors (Wunderlich et al., 2015). Another possibility for EROD inhibition in some fish species is the presence of trace metals, as demonstrated in some studies (Roméo et al., 1994; Guilherme et al., 2008; Vieira et al., 2009). Copper and cadmium have been shown to completely suppress EROD activity in the liver of Trematomus bernacchii (Benedetti et al., 2007). Moreover, the age has also been shown to influence EROD enzyme activity in fish (Whyte et al., 2000; van der Oost et al., 2003; Kashiwada et al., 2007). The influence of age on biomarker response to toxicants is partly the consequence of differences in uptake of contaminants through the life history stage such as larvae, embryos, juveniles and adults (Amiard-Triquet et al., 2013). Furthermore, EROD activity can also be influenced by water temperature (Andersson & Förlin, 1992).

The toxicity of many exogenous compounds can be modulated by induction of phase II biotransformation enzymes, as glutathione S-transferase (GST) (van der Oost et al., 2003). The phase II enzymes modify phase I products into more water-soluble and less toxic forms (Hassan et al., 2015). As EROD, GST activity seems to be correlated to spawning period presenting lower values (Jolly et al., 2012). Nevertheless, the involved mechanisms are not clear. Previous studies have described a bell-shaped pattern in GST activity for different species, where the enzyme activity increases with increasing contaminant exposure until a certain concentration where enzymatic activity progressively decreases (Elia et al., 2003; Vieira et al., 2009). Moreover, both GST inhibition and induction after exposure to different PAH and metals have been reported (Sanchez et al., 2005; Vieira et al., 2009).

A clear gender and seasonal effect has been observed for the biotransformation enzymes described above (EROD and GST), with higher values in male fish compared to females, whereas a decrease in these enzymes has been reported in the bullhead (*Cottus* sp) collected in the summer (Jolly *et al.*, 2012).

Antioxidant enzymes

Many pollutants (or their metabolites) may exert toxicity through oxidative stress. Oxidative stress occurs when the rate of reactive oxygen species (ROS) exceeds the antioxidant defense system. Oxidative stress can derive from increased production of ROS mediated by heavy metals and numerous organic contaminants, including redox cycling compounds, PAHs, halogenated hydrocarbons and dioxins (Livingstone, 2001).

Antioxidant enzymes are commonly employed as biomarkers of oxidative stress; however, responses to

pollution vary from different species, enzymes, and single or mixed contaminants, and even greater variability is found in field situations (Livingstone, 2001). In highly polluted sites, antioxidant enzymes responses can either be induced or inhibited depending on some factors (Fonseca *et al.*, 2011). Habitat, feeding behavior, nutritional status, age and other nonchemical factors have been reported to influence in fish oxidative stress, as well as its antioxidant potential (Ameur *et al.*, 2012; Hook *et al.*, 2014).

Similarly to GST, the enzyme responses to toxic chemicals shows a bell-shaped dose-response curve, with an initial increase in activity due to enhanced catabolic rate and/or direct inhibition by toxic chemicals (Viarengo *et al.*, 2007; Marigomez *et al.*, 2013). This should be taken into account when designing field experiments (Hook *et al.*, 2014).

The reduced activity of superoxide dismutase (SOD) and catalase (CAT) may be attributed to long term exposure of fish to environmental pollutants and a similar type of response has been demonstrated (Bainy *et al.*, 1996; Lenartova *et al.*, 1997). Furthermore, herbivorous fish species have higher SOD activities than omnivorous fish (Radi *et al.*, 1985). Dietary levels of lipids and some vitamins have been reported to influence antioxidant defenses and oxidative status of fish (Ameur *et al.*, 2012). Diets containing oxidized oil significantly affected the activities of liver antioxidant defense enzymes of gilthead sea bream (*Sparus aurata* L.) and dietary vitamin E partially abrogated these effects (Mourente *et al.*, 2002). In another study, activity levels of the antioxidant enzymes CAT and SOD were higher in livers of fish fed with a high lipid level (Rueda-Jasso *et al.*, 2004).

A seasonal variation has been observed for glutathione peroxidase (GPx) activity. This pattern could be observed in a few studies (Sanchez *et al.*, 2007; Jolly *et al.*, 2012), where fish collected in the summer, presented higher GPx activity compared to other seasons, and this could be explained by the involvement of GPx in steroid metabolism (Sanchez *et al.*, 2007).

Cholinesterase (ChE) activity

Among ChE, the acetylcholinesterase (AChE) is the most important, which is found in the nervous tissue and is highly specific to the neurotransmitter acetylcholine (Fulton & Key, 2001; Domingues *et al.*, 2010). AChE removes acetylcholine from the synapses, thus regulating the transmission impulses. Inhibition of AChE in the nervous system results in the accumulation of acetylcholine at toxic levels (Domingues *et al.*, 2010). This results in a larger residence time of the neurotransmitter at the cholinergic receptor, leading to hyperexcitation and death (Printes *et al.*, 2011).

AChE inhibition in many species persists for much longer than pesticide concentration in environmental samples, therefore offering advantage over the use of analytical chemistry alone (Fulton & Key, 2001). Several studies have used this biomarker to evaluate exposure of fish to

organophosphate and carbamate pesticides (Linde-Arias *et al.*, 2008b; Kopecka & Pempkowiak, 2008; Printes *et al.*, 2011; Jolly *et al.*, 2012; Bueno-Krawczyk *et al.*, 2015; Vieira *et al.*, 2016), where its activity is usually decreased. Nevertheless, metallic ions such as arsenic, copper and mercury have shown to inhibit AChE activity as well (Lagadic *et al.*, 1994). Detergents can also lead to AChE inhibition (Guilhermino *et al.*, 1998). High and low AChE activity has been recorded in pesticide polluted sites, reflecting a seasonal variation (Jolly *et al.*, 2012).

A variation in AChE activity has also been attributed to water temperature (Napierska & Podolska, 2005; Kopecka & Pempkowiak, 2008), since it is known that temperature can affect the physiological activity of fish (Bocquenè *et al.*, 1990). However, there are conflicting data in the literature, since some studies have reported a negative relationship between AChE activity and increased temperature in fish (Botté *et al.*, 2013), a positive effect of temperature (Pfeifer *et al.*, 2005; Durieux *et al.*, 2011) or no significant effect (Beauvais *et al.*, 2002; Phillips *et al.*, 2002).

Lipid peroxidation (LPO)

Lipid peroxidation is a well-known mechanism of cell injury in vertebrates and invertebrates, being an indicator of an oxidative damage in cells and tissues. Therefore, measurement of malondialdehyde (MDA), the end product of lipid peroxidation is widely used as an indicator of lipid peroxidation (Wheatley, 2000; Fonseca *et al.*, 2011).

Several studies have shown enhanced LPO in aquatic organisms exposed to high concentrations of metals (Ahmad *et al.*, 2000; Oakes & Kraak, 2003; Pandey *et al.*, 2008; Ruas *et al.*, 2008; Monteiro *et al.*, 2010; Carvalho *et al.*, 2012). Nevertheless, low levels of LPO might be a reflection of feeding habitat and not pollution *per se*, since it has been shown that LPO tends to be lower in herbivorous fish than in omnivorous fish species (Radi *et al.*, 1985).

Heat Shock Protein 70 (HSP70)

HSPs, a family of ubiquitous proteins, are considered the first line of defense following exposure from high temperatures and many other stressors, including xenobiotics and contaminants. Therefore, they are commonly accepted as biochemical indicators of toxicity index, providing a measure of proteotoxicity of pollutants (Kohler et al., 2001; Padmini & Usha Rani, 2008), and thus, this biomarker has been used in some studies (Webb & Gagnon, 2009; Tomasello et al., 2012; Fasulo et al., 2013). The un-modulated expression of HSP70 might be the result of a suppression of the cytoprotective upregulation of molecular chaperone due to either simultaneous exposure to different stressors or high concentrations of pollutants (Ivanina et al., 2009). The HSP70 decrease at high metal concentration might also be attributed to pathological damage and inhibition of protein synthesis in the cell (Clayton et al., 2000; Ivanina et al., 2009).

Although these proteins are involved in the protection and repair of the cell response to xenobiotics and contaminants exposure, it is important to consider their role in conditions such as high or low temperature, ultraviolet light, oxidative conditions, anoxia, and so forth (Stegeman *et al.*, 1992; Di Giulio *et al.*, 1995). It has been reported that HSP70 induction is more suitable as a biomarker of exposure at low temperatures (Triebskorn *et al.*, 1997). Therefore, since nutrition and water quality (pH, temperature, salinity and dissolved oxygen) can affect this protein response, these factors should be considered during monitoring (van der Oost *et al.*, 2003). This biomarker has not been used as much as others in studies over the past years probably because of the difficulty in the interpretation of HPS70 response.

Ecological indexes

Some authors have proposed the use of ecological indexes like gonadosomatic index and condition factor in biomonitoring studies to evaluate the influence of biotic processes or as an additional tool in biomonitoring approaches (Adams & Ryon, 1994), since these indexes are easily obtained. Condition factor values represent a gross index and reflect the condition of the whole body. It is usually related to food quality (Bagnasco et al., 1991). Although it has been used by researchers to provide information on potential pollution impacts (Linde-Arias et al., 2008a; Jolly et al., 2012; Procópio et al., 2014; Bueno-Krawczyk et al., 2015; Ossana et al., 2016), this parameter is not very sensitive and may be affected by non-pollutant factors, such as season, disease and nutritional levels (Mayer et al., 1992). High condition factor values have been found in fish captured in an untreated-domestic-sewage-polluted site (Alberto et al., 2005), whereas no differences were observed in fish exposed to agricultural and urban pressures (Jolly et al., 2012).

Reproductive status is highlighted by the highest gonadosomatic index (GSI). Pollutant concentrations and biological responses showed strong seasonal variations in both mussels and fish, generally related to cyclic physiological changes linked to reproduction and food availability. These results stress the need for careful evaluation of the biological cycles of selected sentinel organisms before planning biomonitoring surveys (Nesto *et al.*, 2007).

CONCLUSION

The use of biomarkers is essential for assessing aquatic ecosystems health. As evidenced in this review, there are some confounding factors that might affect biomarker responses, such as age, gender, feeding habitat and temperature. However, non-pollutant factors are usually only mentioned in the literature; thus, obtaining quantitative data on the extent of the interference of a give non-pollutant factor on biomarker response could contribute to environmental diagnosis. A multibiomarker approach is strongly recommended to reduce data misinterpretation, since

one single biomarker cannot provide a reliable diagnosis of environmental degradation. Furthermore, researchers should also consider the use of unpolluted sites as control areas and collect fish in different periods to monitor possible seasonal variations. The combination of biomarkers with water and sediment physicochemical analysis is also recommended. In this context, laboratory experiments are also invaluable in order to investigate the physiology of a specie to be used as bioindicator in field conditions and mechanisms of toxicity of some substances.

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