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Fish Biomarkers, Suitable Tools For Water Quality Monitoring

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Abstract
A large number of biomarkers and indicator organisms have been suggested for the assessment of ecotoxicity of man-made compounds on aquatic environments. The physiological and biochemical indices in fishes are sensitive for detecting potential toxic effects, and also are obvious from the same reports that studies on the impact of pollutants on the physiological and biochemical status of aquatic organisms. In an attempt to define and measure the effect of pollutants on an ecosystem, biomarkers have attracted a lot of interest. The underlying principle of the biomarker approach is the analysis of an organism's physiological or biochemical response to pollutant exposure. The measurement of biochemical and physiological parameters is a diagnostic tool commonly used in aquatic toxicology and bio-monitoring, so Hematological and immunological parameters are suitable biomarkers in mercury studies. During stress, fish respond in a number of ways in order to regain homeostasis and two important physiological processes which are modulated when fish are exposed to stress, are hormonal status and immune function. In this paper, our previous research's on effects of different pollutants (heavy metals, pesticides, nano particles, organic pollutants and etc.) on many fish species (marine and freshwater) was studied to detect new biomarkers (enzymatic, hormonal, immunological, hematological, histopathological and etc.) for water quality monitoring. In this study we examined markers of hematology, enzyme, hormone and histopathology in different fishes. The aim of this study was to test a multi-trial biomarker approach for evaluating toxicological risk due to the major toxicant in the water, using fishes as bio-indicator organism. The main objectives of this researches were: to identify the tissues and biological materials useful for biomarker studies; to evaluate various biochemical biomarkers in different tissues; to identify the most suitable biomarkers for evaluating chemical stress due to the contaminants explored in this study.

Keywords: Biomarker, Fish, Pollutants, Water quality monitoring.

Origin of biomarkers

A much relied upon means to evaluate ecological risk has been through environmental monitoring in which chemical residues are assessed. This approach has provided useful information but with significant limitations, not the least of which are the time and costs associated with chemical residue analysis, inability to quantitatively evaluate the availability of a chemical from the environmental matrix to the aquatic organism, metabolism or limitations in available technology may render a chemical difficult, if not impossible, to detect in environmental or biological samples.

Application of biomarkers in environmental monitoring may resolve many of these challenges by providing a measure of availability of an environmental chemical to an aquatic organism by providing a direct measure of the response of an organism to chemical exposure. Regarding biological response to sublethal concentrations of environmental chemicals, Depledge (1993) noted that an essential criterion of the biomarker approach is the identification of early onset changes in otherwise healthy organisms that predict increased risk of development of chemically induced pathologies.

During the past two decades, attempts have been made to identify and characterize biomarkers in a range of organisms from bacteria to humans to predict disease or detrimental ecological effects (Adams, 2002; Depledge, 1993).

The term *biomarker* represents many endpoints, and several groups have challenged its original definition. Several definitions of biomarkers have been proposed since the first consensus definition proposed by the Committee on Biological Markers of the National Research Council (NRC) (1987). The NRC defined biomarkers as “indicators signaling events in biological systems or samples following chemical exposure” and proposed the use of biological markers to determine: (1) internal dose or biologically active concentration (exposure), (2) adverse effects, and (3) susceptible populations or individuals in an attempt to predict and possibly prevent clinical disease, specifically in humans. In fact, in the original definition and classification by the NRC (1987), the emphasis was placed on human health, specifically associated with reproductive toxicity. With fish specifically in mind, Adams (2002) modified the original NRC definition to include characteristics of organisms, populations, or communities that respond in measurable ways to changes in the environment. As the measurements have proceeded to include other organisms such as fish, debate has occurred as to their utility as a “marker” or as an “indicator” in ecological settings (McCarty and Munkittrick, 1996). It has been further argued that studies examining a biological response without a definitive purpose are essentially useless as “indicators” (Holdway, 1996). Peakall (1992) suggested the term *Biomarker* to indicate effects relating to individual organisms and *bioindicator* to indicate effects measured at the population or community levels of biological hierarchy. It is clear from the multiple definitions of the term *biomarker* that any study using this terminology must begin by defining the specific aims and purposes of the biological response that is measured or proposed as a biomarker.

The NRC proposed three types of biomarkers in an attempt to classify responses as markers of exposure, effect, and susceptibility. Each of these definitions has been addressed previously and discussed in terms of its potential use in ecological risk assessment paradigms (Schlenk, 2006). As more biomarkers have been increasingly proposed and characterized, significant overlap may occur when using this nomenclature, as some biomarkers can be in each of the three capacities. An effect resulting from stressor exposure may be defined as an early adaptive nonpathogenic event or as a more serious altered functional event, depending on the toxicokinetics and mechanism of action of the compound (Decaprio, 1997). Likewise, biomarkers of exposure and effect may often be combined into a single classification, with susceptibility occurring along any stage (Barrett et al., 1997).

In recent decades, aquatic toxicology has moved from a descriptive approach, which was necessary to explore those concentrations of single toxicants within water that were not compatible with the life of individual fishes, to considerations of sublethal concentrations that do not cause death over the short term but do harm the individual, thus making it expend resources to survive in a state of altered equilibrium and these helped to cut across questions of bioavailability as the emphasis shifted to host response (Di Giulio and Hinton, 2008).

One of the benefits of the biomarker approach is the identification of early-onset changes, which predict increased risk of adverse effects following exposure to environmental chemicals. There are many definitions of biomarkers e.g.: "A biomarker is a xenobiotically induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample" (NRC, 1987).

Different type of biomarker

Toxic effects or responses can be divided into those that are "graded" and those that are "all or none". Graded effects are those such as the inhibition of an enzyme which can show some effect between zero and maximal. All-or-none responses are those that are only present or absent (on or off), such as death or the histopathologic abnormality (Timbrell, 2009).

The IPSC has three classes of biomarkers identified: biomarkers of exposure of the organism to the toxic substance, biomarkers of response of the organism to that exposure, and biomarkers of susceptibility of the organism to the chemical (WHO, 2005).

Biomarkers of Exposure: At its simplest, measurement of the dose is determination of the amount of chemical administered or the amount to which the animal is exposed. The level of a chemical in the blood approximates to the concentration in organs and a tissue is a true biomarker of exposure. Biomarkers of exposure are relatively transient and generally only detectable for about three months after exposure. However, a metabolic breakdown product may be responsible for the toxicity, and therefore, measuring the parent chemical may not always be an appropriate biomarker of exposure. Biomarkers of exposure are important in risk assessment, as an indication of the internal dose is necessary for the proper description of the dose-response relationship (Timbrell, 2009).

Biomarkers of Response: Living organisms can show many kinds of toxic or adverse response to a chemical exposure, ranging from biochemical or physiological to pathological. Consequently there are many biomarkers of response, which can be measured. These include markers such as enzymes, which appear in the blood when an organ is damaged and pathological changes. Indeed, a biomarker of response could be almost any indication of altered structure or function. However, although the new technologies (genomics or transcriptomics, proteomics and metabonomics) have an increasingly important role, interpretation of the often large amount of data generated is a significant task requiring bioinformatic techniques such as pattern recognition. Furthermore, all biomarkers of response must be validated in relation to certain criteria. It cannot be assumed, because a gene is switched on or off. Biomarkers of response are necessary for determination of the no observed adverse effect level (NOAEL) and the dose-response relationship (Timbrell, 2009).

Biomarkers of Susceptibility: these biomarkers cover a range of types from deficiency in metabolic enzymes to variation in repair systems. These would typically be measured in individual members of a population. An example could be a genetic deficiency in a particular enzyme involved in detoxication or xenobiotic metabolism. Biomarkers of susceptibility may be important for identifying especially sensitive groups to estimate an uncertainty factor (Timbrell, 2009).

Development of the Biomarker approach

The measurement of various contamination of sea water by classic chemical monitoring of few pollutants is possible, but also through examination for indicators of adverse effects of pollution on organisms is more effective. Selected biochemical parameters, so called biomarkers in an indicator fish, can be used for this purpose (Van der Oost et al, 2003).

Chemical monitoring of any persistent toxicant concentration in marine water as well in sediment may not provide accurate data on the stringency of contamination, especially in the case of natural condition, But biological monitoring using a series of assays having different endpoints in a key species could allow a sensitive approach to predict the potential risk of persistent contaminants like heavy metals.

In last decade, the use of biomarkers for many monitoring of marine environment has become wide spread. Biomarkers have been largely used for the assessment of effects induced by several classes of chemical contaminants on fishes, for example the assessment of alterations on some enzymatic activities of key species following exposure to natural and experimental contaminated waters has been one of the major uses of biomarkers in marine studies. Livingstone (2001) shows the main idea for the future evolution and purpose of the biomarkers and reports the role of biomarkers of oxidative stress in Ecotoxicology. Biomarkers have basic requirements for ecotoxicological study, such as: fast responsibility; low cost; simple procedures; applicability under varied testing conditions both in environment and labratory; and sensitivity to a high number of environmental contaminants include heavy metals.

In trying to define and measure the effect of pollutants on marine environment, biomarkers have been more interest. The doctrinaire principle of the biomarker approach is the assay of an organism's physiological or biochemical response to pollutant exposure, because toxic effect explicit itself at the sub cellular level before it becomes apparent at organ levels of biological organization. The measurement of biochemical responses to heavy metals will improve the assessment of biologically significant exposures to toxic metals and enhance the ability to evaluate the effects of xenobiotics on the health and survival of toxicant exposed fishes. With compare to direct monitoring method, biomarkers have the advantage of being more relevant biologically (Rees, 1993). Scientific studies for the identification of marine pollution biomarkers have been carried out extensively in animals in general and then fish in particular (Oikari and Jimenez, 1992).

A combination of physical, chemical and biological indicators is frequently used to evaluate water pollution (Karr, 1993). But in last decade, there has been a growing awareness of the need to detect and evaluate the effects of pollution in living organism (Schlenk, 2006). Heavy metal concentrations in aquatic organism are often more than aquatic environment in which the organisms resides. This suggests that organisms can be used as biological markers of metal pollution (Flessas et al., 2000).

Fish are largely being used for evaluate of water toxic metals and can serve as bioindicators of environmental pollution. The ability to accuracy predict of the bioaccumulation of toxicant in fish has become an essential component in evaluation of the ecological and human risks in exposure to toxic pollutants, and also such estimates are needed to assess more accurately potential ecological risks to fish assemblages themselves. The long time exposure of fish to toxicants results primarily accumulation in their organs and tissues and secondly in sub cellular alterations due to their continuous deleterious action.

Although exposure-referenced toxicological benchmarks such as the LC_{50} and the EC_{50} have been widely used to make toxic evaluation, most harmful effects of chemical pollutants are because of accumulation of those compounds, more than their environmental concentrations per se (van Loon et al. 1997).

A range of different biomarkers have been used to indicate the biological effects of certain pollution on fish, both in natural environments and under experimental conditions (Ferrando et al., 2006).

The historical development of the biomarker approach can be seen to have close links with medicine and vertebrate biology (N.R.C, 1987). However, biomarker measurements are completely possible in invertebrate organisms (Depledge, 1993). There are several reasons why studies on fishes are better for ecological risk assessment. For example, fishes constitute more than 28000 species, they are major components of all ecosystems, and fish populations are often numerous, so that samples can be taken for analysis without significantly affecting population dynamics. Increasing knowledge of the physiology of fishes now permits reasonable interpretation of biomarker responses in terms of ecological risk assessment.

Application of Biomarkers

The release of different type of wastes generated by modern human activities into marine ecosystems potentially induces various combinations of stresses to fish. The evaluation of environmental xenobiotics requires explain of stress effects throughout the hierarchy of biological organization, from molecular and cellular levels up to organism and population levels (Moore, 2002). Therefore the assessment of different biomarkers to investigate the in vivo and in vitro effects of contaminants is a priority requirement to reveal the action mechanisms of xenobiotics. As marine ecosystems are the mostly final receptacles of industrial and urban waste discharges (Hoffman et al., 1995), a fundamental goal in ecotoxicology is to risk assessment of toxicant for aquatic organisms and human wildlife.

The investigation of the proper biomarkers for the best possible diagnoses is very important for researchers. Environmental experiments involving the use of biomarkers are recognized as one of the most powerful tools for the investigation of pollutants (Depledge, 1993).

Water pollutants cause toxic effects that range from biochemical alterations in single cell to the changes in organ and even whole population on aquatic organisms (Bernet et al., 1999). As the fish are considered as one of the bioindicator of the water contaminant, the extent and effect of pollution in them can be monitored by examining the sublethal indices or biomarkers.

Regarding to the biological effects of toxicant, the biomarker approach can offer more complete and relevant information regarding the potential impact of contamination on the health of an animal (Van der Oost et al., 2003). Exposure to environmental toxicants can launch the activation of defense mechanisms, the performance of which determines the toxic effect on the organism (Blaise et al., 2002).

The evaluation of physiological effects of chronic and sub chronic metals exposure using biomarkers of sublethal toxicity are necessary in order to assessment the impact of pollution under realistic conditions. These effects include genotoxic (Sanchez-Chardi and Nadal, 2007), enzymatic (Swiergosz- Kowalewska et al., 2006), haematological (Rogival et al., 2006), and histological alterations (Pereira et al., 2006) that in general only occur when substantial concentrations of metals are present in the tissues and blood. In fact, the combined use of biomarkers with bioaccumulation data provides a suitable measure of health status, physiological condition, and response of Fish populations to contaminant.

With complex environmental xenobiotics, it is ineffective to quantify all the contaminants. In this context, a strategy involving biomarkers has been demonstrated to be a suitable alternative for monitoring and management of whole aquatic ecosystems (Flammarion et al., 2002). Biomarkers enable the assessment of probable contaminent, in the environment, since chemicals may behave differently when acting individually or in mixtures. Besides, biomarkers reduce expensive cost of chemical analyses and the information provided may be used as an early warning system (Marin and Matozzo, 2004). Biochemical and physiological biomarkers, in particular, have been used in order to arrest irreversible damage in whole organisms, communities and ecosystems (López-Barea and Pueyo, 1998).

Biochemical biomarkers are frequently used for detecting or diagnosing sublethal effects in fish exposed to toxic substances. Choose of the suitable biological effect markers for the study of the chronic and sub chronic contaminant is frequently a controversial issue, when information on the mechanism of action of the contaminant is incomplete. But even very low exposure to contaminants may cause various biological effects (Toguyeni et al., 1997).

The application of biomarkers for mercury toxicity is of most interest to monitor not only the presence of mercury in the animal body, but also its bioavailability and capacity to find biological responses. Many biomarker studies examine the effects of toxicant exposure on whole soft tissue homogenate, but this approach may not always be suitable, as the partitioning of compounds among different tissues may largely influence toxicity and may be masked when measurements are restricted to whole body concentrations (Depledge and Rainbow, 1990). For example different body tissues have various abilities to accumulate metals (Gundacker, 1999), therefore using a suitable biomarkers from a blood that is current in whole body can produce more specific and relevant results to making an actual face of what is occurring in the ecosystem.

Biomarker approach measure directly the concentration of a toxin in an organism, not accounting for the biological effect, that it is one of the most important advantages of biomarkers (Depledge, 1993). For this reason a biomarker study was undertaken using in vivo and in vitro *A. latus* as a key species.

In the present study chemical analysis (of mercury) and three types of biomarker responses were investigated: (a) biomarkers of defense or early biological effect, (b) biomarkers of damage and (c) biomarkers of reproduction. The above mentioned biomarkers were selected because they have a definite role for the survival and performance of fish under heavy metals stress.

Correlation of biochemical and structural analyses in labratory, as well as *in vivo* exposures, led to the production and application of biomarkers of exposure and effect wild fishes. Resultant biomarkers were applied to heavily contaminated and reference field sites as part of effects assessment and in investigations following large-scale disasters such as oil spills or industrial accidents.

Clearly, to avoid misinterpretation of biomarker responses, mechanistic links by which chemical effects at one level of organization give rise to detrimental effects at higher levels of biological organization must be established. As an example, alterations in steroid metabolism resulting in changes in hormone profiles which, in turn, alter sexual behavior might consider as chemical effect. So specific understanding of the normal homeostatic roles for these mechanisms should be achieved prior to their use as biomarker and also in the design of biomarker strategies, an integrated approach should be considered in which a hierarchy of responses are evaluated. The hierarchy can be constructed based on the level of biological organization that is being monitored or on different degrees of response sensitivity.

Development of the Biomarker approach

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Application of Biomarkers

The investigation of the proper biomarkers for the best possible diagnoses is very important for researchers. Environmental experiments involving the use of biomarkers are recognized as one of the most powerful tools for the investigation of pollutants (Depledge, 1993). In the biomarker approach it is either the activated defense mechanisms or the toxic effect that is measured in an organism, or both.

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Enzymatic Biomarkers

Chronic pollution induced heavy metals in the marine ecosystems is a major problem particularly in shallow water like creeks. Heavy metals may modify the structure of the cell membranes by stimulating the lipid peroxidation process concentration with consequent complex sequences of biochemical reactions (Viarengo, 1985), This process is generally known as oxidative Research deterioration of polyunsaturated fatty acids. In fishes generaly, peroxidation of lipids cause to the production of lipid radicals and in the formation of a complex mixture of lipid degradation products including malonyldialdehyde and other aldehydes such as alkanals, hydroxyalkenals and ketones hepatopancreas, (Viarengo, 1985).

Heavy metals accumulated in the fish tissues may catalyze reactions that generate reactive oxidative species (ROS) which result to environmental oxidative stress. These systems contain different antioxidant defenses. Defensive mechanisms to discomfit the impact of ROS are found in many species including aquatic animals such as fish.

In spite of seriousness and longevity of heavy metals in the ecosystem, that they are non-degradable with significant oxidizing capacity and substantial affinity for electronegative nucleophilic species in proteins and enzymes.

Enzymes catalyse physiological reactions by decreasing the activation energy level that the reactants (substrates) must reach for the reaction to occur. The influense of pollutants on enzymatic activity of fish is one of the most important biochemical parameters which is affected under exposure of toxicants. In exposure to a toxicant, enzyme activity appears to be increased or it may be inhibited due to the active site being either denatured or distorted. Since some enzymes catalyse some steps in the metabolism of carbohydrates and protein, they are present in most tissues. The increase or decrease in enzyme level in a very accurate index for diagnostic of quantity and quality of toxicant. For example, such effects have been observed after chronic exposure to low doses or acute exposure to high doses of mercury.

Similar research on fish enzymes have demonstrated that antioxidant systems could provide relevant indices in explaining the sensitivity of some fish species to pollutions (Di Giulio et al., 1993). Antioxidants have a very sensitive role in maintaining cell homeostasis and, when these defenses are impaired or surmounted, oxidative stress products, namely reactive oxygen species (ROS), may induce DNA damage, enzymatic inactivation and peroxidation of cell constituents. Fish often increased the levels of protective antioidxants enzymes, as well as non-enzymatic free radical scavengers for prevent and cope again abnormality that cause by ROS. Thereupon, one of the suitable biomarker of exposure to heavy metals is the modulation of antioxidants enzymes for example mercury was recognized as a pro oxidant that induces oxidative stress (Stohs and Bagchi, 1995).

Induction of oxidative stress causes with mercury make an important contribution to molecular mechanism for liver injury. Recent studies confirm that mercury causes severe oxidative damages (Kim and Sharma, 2005) thus mercury is proved to be a potential oxidant in the category of environmental factors.

Hematology and Immunology Biomarkers

The finding of suitable biomarkers for the best possible diagnoses is very critical for ecotoxicological studies. Blood indices are considered pathophysiological parameters of the whole body and therefore are important in diagnosing the structural and functional status of fish exposed to xenobiotics (Adhikari et al., 2004).

Moreover, hematological indices provide quite frequently and routinely accepted methods in aquaculture to evaluate the interactions between dietary levels of nutrients (Lim et al., 2000). Although fish blood indices have been increasingly examined in ecosystem monitoring programs as valuable parameters of physiological changes in the presence of xenobiotics, the lack of basic knowledge about the blood response to stressors mainly from tropical species is the most important leakage to using these indices in environmental monitoring programs (Affonso et al., 2002).

The intensity and duration of these responses and/or effects are effected by several factors, including the concentration of the contaminant, duration of exposure, and the fish species (Heath et al., 1995)

The measurement of biochemical and physiological parameters is a diagnostic tool commonly used in aquatic toxicology and biomonitoring. Hematological parameters are more often used when clinical diagnoses of fish physiology are used to determine subchronic concentrations of pollutants.

Physiological changes induced by xenobiotics are also apparent at the biochemical and physiological level, such as in the carbohydrate and protein metabolism and in hematology. In cases where these alternations are adaptive they are referred to as stress responses, while they are considered effects when they have a negative cause on the physiological condition or even survival of the fish. The intensity and duration of these responses and/or effects are effected by several factors, including the concentration of the contaminant, duration of exposure, and the fish species (Heath et al., 1995). Other research have confirmed this found, for example, changes in hematocrit, hemoglobin, plasma glucose, and lactate levels in Cd-exposed fish (Gill and Epple, 1993). Although the immunotoxicity of mercury is well established, evaluation of their potential immunotoxicity in marine biota is complicated by variables that could modulate the immune response to contaminants under field conditions.

Hormones Biomarkers

Thyroid hormones (THs) have many physiological roles in fish like growth regulation, development, metabolism and hydromineral balance (Van Anholt et al., 2003). A little change in serum concentrations of these hormones, as well as in glucose levels reflects endocrine changes; thereupon, fish physiological competence to cope with ecosystem xenobiotics can be affected. Thus, the hormones biomarkers may also be useful tools in monitoring the impact of heavy metals stressors on fish. Also HPT alterations provide useful data about the health status of fish, being reliable candidates as biomarkers of ecosystem stressors (Teles et al., 2005).

During chemical exposure, for regaining safe homeostasis fish do much physiological processes and two important physiological processes which are modulated when fish are exposed to stress, are hormonal status and immune function (Wendelaar-Bonga, 1997). Whereas it is conspicuous that both of these processes are necessary for an animal survive, but there is few knowledge about role of hormone biomarkers during mercury exposure of marine fish, so in this study, a multi factorial approach, involving determining thyroid hormones as well as measurements of parameters of the non specific immune response like glucose, during the in vivo and in vitro exposure of mercury chloride was used. The information gained from this study may be useful for future strategies in monitoring and predicting the effects of mercury exposure and also in developing indices to measure stress during sea bream culture.

Endocrine Disruption Biomarkers

Environmental pollution by endocrine disruptors is presently a growing awareness concern. Such man made chemicals can mimic or block hormones interfering with the endocrine system and finally compromising crucial biological processes. The increasing hazardous of xenobiotics on biota and making potential endocrine disrupting is a serious threat to human and wildlife health. (Morgado, 2007).

The endocrine procedures have a specific role in fish stress mechanisms. Thus, we can use any changes in specific hormonal functions and consequent biochemical effects as important stress biomarkers.

Based on similar study related to freshwater fish, impacts of contaminants on sex steroid titers might be expected in marine fish, but few have been reported to date. In principle, sex steroids alternation in fish serum is because of intervention with the control of steroid synthesis via the pituitary-gonadal axis, or to effects on steroid metabolism and excretion (Matthiessen, 2003).

In animals sex steroid hormones are produced by the endocrine system and control the life cycle stages of an organism including gametogenesis, fertilization, sexual development, and reproduction. Recent studies have established that a wide variety of man made chemicals in the ecosystems are capable of modulating and adversely affecting or disrupting endocrine function in animals (Tyler et al, 1998).

Histopathology Biomarkers

Histopathology is now recognized as useful index to assessment the effects of toxicants in vital processes such as growth and reproduction, detecting early effects of pollutant in cells, tissues and organs. Histopathological biomarkers have been widely used in fish for detection and assessment on chemical effects of exposure to toxicants. Also histopathological indices have been largely used as biomarkers in the monitoring of fish health status during exposure to toxicants, both in the laboratory and field studies (Thophon *et al.,* 2003).

Histopathological biomarkers allows examining specific target organs, including gills, gonad and liver, that are responsible for vital functions, such as respiration, reproduction and the accumulation and biotransformation of xenobiotics in the fish and this fact is very important advantage of these category of biomarkers in monitoring programs of marine ecosystems. Moreover, the changes detect in these organs are normally easier to identify than functional ones, and serve as warning signs of damage to animal health (Hinton & Laurén, 1990).

Fish tissues are sensitive indicators of marine toxicant and have a high mercury bioaccumulation capacity for both organic and inorganic forms solution in marine environment. Recent studies have confirmed links between exposure to pollutants and the development of hepatic lesions. For example toxicopathic liver lesions in fish species are suitable and sensitive signs of toxicant-induced injury and have been used as biomarkers of chemicals in environmental risk assessments. Hypertrophy of the liver is a common response of teleosts to pollutants and is linked to hepatic detoxication mechanisms (Lemaire et al., 1992).

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