Use of Molecular, Biochemical and Cellular Biomarkers in Monitoring Environmental and Aquatic Pollution

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Abstract: This paper gives an overview of the variety of animals and plants that are available for use as environmental and aquatic pollution monitors. Most aquatic and environmental management authorities required assessing the potential toxicity of metals-contaminated effluent at its point of discharge to avoid the determining effects of toxic metals in high quality food. Otherwise changes in fish health due to pollution may decline in fish population. Understanding the effect of toxicants on fish innate immunity supports the larger ecotoxicological goal of comprehending the actions of ecotoxicants on fish populations. There have been various reports that the utilization of a single species or target biomarker is not appropriate or scientifically sound for the monitoring of several toxic pollutants. Recent years have seen the development of biological measurements (biomarkers) as tools for use in monitoring and environmental impact assessment, such biomarkers being indicative of contaminant exposure and/or impact. The results of the study by most researchers indicated that biological effect monitoring is the only appropriate method providing a reliable environmental risk assessment. The advances in molecular genetics have led to an upsurge in interest in most susceptibility factors, and identification of polymorphisms of various enzymes has become possible. Among various biochemicals, cellular and physiological systems, certain innate immune responses are considered as suitable biomarkers for monitoring biological effects of pollution. Ongoing search for "ultra-high risk" individuals may be fruitful, but probably only relevant to a small segment of potentially exposed populations. The monitoring efficacy can be greatly improved by using batteries of non-specific biomarkers comprising different biological levels. Thus, the use of molecular, biochemical and cellular biomarkers has proved to be very useful in environmental and aquatic pollution monitoring.
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1. Introduction

In the last few decades, a possible influence of environmental pollution on the aquatic environment has gained considerable interest (Skouras et al., 2003). Molecular, biochemical, cellular and immunological markers have been extensively used in pollution monitoring of aquatic environments. Biochemical markers have been selected among early molecular events occurring in the toxicological mechanisms of main contaminants (Banni et al., 2005).

The term biomarker refers to the physiological, biochemical, and histological changes used as indicators of exposure to chemical contaminants and/or of its effects at the suborganismal or organismal level. Recently the development of sensitive biochemical markers for monitoring environmental quality in aquatic ecosystems has raised a great deal of interest (Pretti and Cognetti-Varriale, 2001). Selected biochemical markers measured in feral organisms can provide sensitive indexes, or early warning signals, of potential ecosystem degradation caused by contaminants. Compared with chemical residue analysis, biomarkers have the advantage of measuring the stress on the organism, thus being more biologically relevant (Pretti and Cognetti-Varriale, 2001). The use of cholinesterase activity as a biomarker of pesticide exposure for biomonitoring in estuarine areas is also given as an example (Pretti and Cognetti-Varriale, 2001).

Biomarkers are promising tools for biomonitoring, both in the marine and freshwater environment. It is however clear that much more information is needed about the exact relation between biomarker responses and the health and fitness of organisms, and even more so between biomarker responses and risks for the ecosystem. In order to address these questions, it is important to realise that the possibilities for the application of a biomarker depend on the concept that is chosen for environmental monitoring. The purpose of monitoring programs varies from simple screening to risk

characterisation on the ecosystem level (den Besten, 1998).

Fish have become a favourable subject for research in this area, because temperature changes, habitat and water quality deterioration as well as aquatic pollution adversely affect fish health, which may result in mortalities and population decline (Skouras et al., 2003). Among various biochemicals, cellular and physiological systems, certain innate immune responses are considered as suitable biomarkers for monitoring biological effects of pollution (Bols et al., 2001; Skouras et al., 2003).

Heavy metals concentration in the tissues of fish enter into human beings through food chain and due to there cumulative action causes potential health hazards sometimes even lethal (El-Shehawi *et al*., 2007). The toxic effects may result from the bio concentration of metals and their consequence binding with biologically active constituents of the body such as lipids, amino acids and proteins (Smedes and Thomson, 1996; Thangam and Sivakumar, 2004; Vutukuru, 2005).

Chuddar *et al*. (2002) report that heavy metal, nickel effects biochemical component like glycogen of gill, digestive gland and whole body of freshwater bivalve, *Parreysia cylindrical* under studied. The significant decrease in total glycogen content of gill, digestive gland and whole body was observed due to pollution stress caused by nickel (Azmat et al., 2008).

Impairment of immune functions, which protect fish against invading pathogens, can lead to harmful consequences at the individual level, such as disease outbreak followed by death, and at the ecosystem level, as population reductions are followed by changes in the entire ecosystem (Skouras et al., 2003). For fish populations, a link between environmental pollution and diseases has long been expected, and studies carried out under defined laboratory conditions concluded modulating influences of xenobiotics on fish immune responses (Bols et al., 2001; Skouras et al., 2003). Understanding the effect of toxicants on fish innate immunity supports the larger ecotoxicological goal of comprehending the actions of ecotoxicants on fish populations (Bols et al. 2001; Skouras et al., 2003).

From laboratory studies, it has become clear that environmental contaminants indeed modulate immune responses in fish (Bols et al., 2001; Skouras et al., 2003). However, with attempts to extrapolate experimental data to 'field' situations in monitoring studies, problems arise (Skouras et al., 2003). In natural environments, fish are exposed to an undefined cocktail of various substances for an unknown period of time (Skouras et al., 2003). To gain knowledge about the actual contamination level of the individuals studied, the study by Skouras et al. (2003) was substantiated by extensive analytical chemistry of sediment as well as fish residues, which included chlorinated hydrocarbons and heavy metals (Dizer et al., 2003).

It also has to be taken into account that contaminants may cause indirect effects such as elevated levels of cortisol, which have a marked modulatory potential on immune functions (Skouras et al., 2003). Stimulatory effects of contaminants, as observed in the study by Skouras et al. (2003), may also be a consequence of indirect effects of contaminants. Some contaminants, however, such as copper, are clearly immunotoxic. Cellular immune responses are considered to be sensitive indicators of biological effects of pollutants (Skouras et al., 2003).

In this review, we describe some of the most frequently used biomarkers in aquatic biomonitoring in different countries. It also reviews the potential of biomarkers for ecotoxicological status assessment in biomonitoring programs. For this purpose, we define the roles and the functions of biomarkers as biomonitoring tools. We also highlight the importance of defining a clear reference system to be confident that biomarkers represent a quantitative assessment of the effects of contaminants. This paper compares a number of these concepts with respect to how biomarkers can be used and with respect to the specific requirements for further implementation of biomarkers in environmental monitoring (den Besten, 1998). This paper also presents an overview of the significance of the use of molecular biomarkers as diagnostic and prognostic tools for marine pollution monitoring.

2. Historical Background of biomarkers in monitoring aquatic pollution

A thorough, critical review of the literature shows that over two decades ago international and domestic symposia resulted in preliminary recommendations for establishing formalized monitoring programs in marine and estuarine habitats (Pearce and Despres-Patanjo, 1988). Early on, enforcement and management agencies emphasized the need for development of techniques that would be useful in compliance monitoring. Efforts were initiated to insure that management and pollution abatement programs resulted in reduction of contaminant loading (Pearce and Despres-Patanjo, 1988).

In the early 1970s managers concerned with aquatic life, especially important commercial species emphasized the need for biological effects monitoring, which was designed to indicate contaminant effects, and changes in degree of effects with time, on living

resources. Strategies subsequently were developed for long-term effects monitoring to demonstrate how changes in contaminant distribution and abundance might affect aquatic life (Pearce and Despres-Patanjo, 1988).

Over the past decade, molecular, biochemical and cellular markers have been extensively used in pollution monitoring of aquatic environments (Banni et al., 2005). Organic contaminants are continually entering aquatic environments and thence the tissues of resident biota. Mussels and other molluscs are used worldwide as sentinels in pollution monitoring (Livingstone et al., 2000). Metals and organic contaminants, present in the water-column, sediment or food, are readily accumulated by aquatic organisms (Livingstone, 1993).

2.1. Development of biomarkers

In the past 25 years, numerous biomarkers have been developed with the objective to apply them for environmental biomonitoring (Sanchez and Porcher, 2009). Recent years have seen the development of biological measurements (biomarkers) as tools for use in monitoring and environmental impact assessment, such biomarkers being indicative of contaminant exposure and/or impact (Livingstone et al., 2000). At about the same time various agencies began to stress the importance of tracing sources and fates as well as the effects of contaminants as they are introduced into aquatic ecosystems, especially estuaries where effects have been most strongly observed. High priority was placed on understanding the fates of contaminants and the consequences of physical degradation as tidal waters moved materials from riverine systems through estuaries to coastal zones. The NOAA National Status and Trends Program (NS&TP) and the Northeast Monitoring Program (NEMP) are examples of the most recent strategies. More recently, the U.S. EPA, FDA, FWS and NOAA have stressed the development of hazard or risk assessments in regard to inorganic and organic contaminants and gross categories of complex wastes (Pearce and Despres-Patanjo, 1988).

Recently, the Water Framework Directive (WFD) of the European Union specified monitoring programs required to assess the achievement of good chemical and ecological status for all water bodies by 2015. This article reviews the potential of biomarkers for ecotoxicological status assessment in WFD monitoring programs. For this purpose, we define the roles and the functions of biomarkers as biomonitoring tools. We also highlight the importance of defining a clear reference system to be confident that biomarkers represent a quantitative assessment of the effects of contaminants (Sanchez and Porcher, 2009).

2.2. Recent molecular, genotoxic, cellular and immunological studies on the development of biomarkers

A large variety of environmental carcinogens are metabolically activated to electrophilic metabolites that can bind to nucleic acids, forming covalent adducts (Vanschooten et al., 1995). In organisms possessing active metabolic systems for a particular carcinogen, DNA adducts generally have longer biological half-lives than the substrate carcinogens (Vanschooten et al., 1995). Thus, measurement of specific DNA adducts concentrations in terrestrial and water organisms may provide a relevant biological indicator of prior exposure to environmental carcinogens (Vanschooten et al., 1995). Analysis of carcinogen load in indicator species with specific behavioral patterns by may indicate human exposure risk to environmental carcinogens (Vanschooten et al., 1995).

Recently, sensitive assays have been developed to measure carcinogen-DNA adducts in organisms exposed to complex mixtures such as polycyclic aromatic hydrocarbons (PAH). At first instance, the nuclease P1 version of the ^{32}P-postlabeling assay was used by Vanschooten et al. (1995) to examine the liver of eel (*Anguilla anguilla*) for the presence of aromatic DNA adducts. The fish were collected from six freshwater sites in the Amsterdam area with different levels of PAH contamination in their sediments. Chromatograms derived from DNA of fish from polluted sites revealed a broad diagonal zone indicating the presence of DNA adducts containing aromatic or bulky hydrophobic moieties not present in DNA of fish from an unpolluted reference site (Vanschooten et al., 1995).

Significant correlations were found between the aromatic DNA adducts levels and the levels of PAH in sediments ($P < 0.001$) in the study by Vanschooten et al. (1995). Several aromatic DNA adducts could be detected in DNA from the exposed earthworms; adduct levels were significantly increased with increasing exposure time (Vanschooten et al., 1995). The findings by Vanschooten et al. (1995) suggested that the amount of DNA adducts in eel and earthworm may be a suitable and sensitive indicator for the exposure to carcinogenic PAH from contaminated sediments or soils, respectively, and therefore useful in human exposure risk assessment.

Organic contaminants are continually entering aquatic environments and thence the tissues of resident biota. Mussels and other molluscs are used worldwide as sentinels in pollution monitoring (Livingstone et al., 2000). Recent years have seen the development of biological measurements (biomarkers)

as tools for use in monitoring and environmental impact assessment, such biomarkers being indicative of contaminant exposure and/or impact (Livingstone et al., 2000). Livingstone et al. (2000) described established and developmental biomarkers in mussels responsive to exposure to organic contaminants, including some indicative of damage to DNA ("comet" assay) putative induction of biotransformation enzymes (CYP1A-like protein), contaminant removal (MXR-like protein), lysosomal membrane damage and impairment of membrane function.

3. Types, Advantages and Importance of biomarkers in monitoring aquatic and environmental pollution

3.1. Different Types of Biomarkers

Different types of biomarkers included: molecular, biochemical, cellular and immunological biomarkers. The primary aim of a study by Van Der Oost et al. (1996) was to select a set of relevant biomarkers in feral eel for the biological assessment of inland water pollution. In a study by Van Der Oost et al. (1996), hepatic activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and GSH-peroxidase (GPOX)) in eel did not show any response to pollution and are therefore not feasible as biomarkers. The reduced glutathione (GSH) cofactor levels in eel liver are most probably not reliable as biomarkers (Van Der Oost et al., 1996).

Jamil (2001) provided an excellent overview of the variety of animals and plants that are available for use as environmental monitors. According to Jamil (2001), the utilization of a single species or target bioindicator is not appropriate or scientifically sound for the monitoring of several toxic pollutants. This is an important concept. The excellent overview of frequently found toxic pollutants in the environment provided firm toxicology foundation for subsequent examination of specific biomarkers and mechanisms of toxic agent activity.

Among the various types of biomarkers, the following have received special attention: cytochrome P4501A induction, DNA integrity, acetylcholinesterase activity and metallothionein induction. A suite of biochemical parameters in eel (hepatic biotransformation enzymes and cofactors, antioxidant enzymes, PAH metabolites, DNA adducts, serum transaminases) has been measured in order to determine their response to xenobiotic compounds in the environment (Van der Oost et al., 1996, 1997). The activity of UDP glucuronyl transferase (UDPGT) in eel may, however, be a useful biomarker (Van Der Oost et al., 1995, 1996). The level of 1-hydroxypyrene (1-OH pyrene) in eel bile might be a useful biomarker to determine short-term PAH exposure (Van der Oost et al., 1996, 1997). The hepatic level of DNA adducts in eel liver seems to be a sensitive biomarker for exposure to (and possible effects of) mutagenic and carcinogenic xenobiotics (Van Der Oost et al., 1995, 1996, 1997). The proposed set of the most relevant biomarkers for the assessment of inland water pollution with feral eel thus consists of the following six parameters: $cytb_5$, CYP1A, EROD, EROD/P450, UDPGT and DNA adducts (Van Der Oost et al., 1995, 1996, 1997).

Organic trace pollutants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polycyclic aromatic hydrocarbons (PAHs). In addition, the pollution-induced responses of a suite of 21 biochemical parameters in eel (notably phase I and phase II biotransformation enzymes, antioxidant enzymes, PAH metabolites, DNA adducts and serum transaminases) were measured in that study by van der Oost et al. (1997) as biochemical biomarkers.

Measurement of the induction of cytochrome P4501A in terms of EROD (7-ethoxy resorufin *O*-deethylase) activity is successfully used as a potential biomarker of exposure to xenobiotic contaminants in marine pollution monitoring (Sarkar et al., 2006). The evaluation of acetylcholinesterase activity in marine organisms has been used as a biomarker of exposure to neurotoxic agents such as organophosphorus, carbamate pesticides etc and to assess the impact of neurotoxic compounds on marine environment (Sarkar et al., 2006). Metallothioneins (MTs) are induced by toxic metals such as Cd, Hg, and Cu by chelation through cysteine residues and are used in both vertebrates and invertebrates as a biomarker of metal exposure (Sarkar et al., 2006).

Diethylstilbesterol has been used as a model estrogen and/or positive control in a number of toxicologically-oriented studies with fish [e.g., Folmar et al., 2002; Panter et al., 2002; Zhong et al., 2005; Yang et al., 2008; Adedeji et al., 2012). However, this work has not identified no-effect water concentrations of the estrogen. For example, Panter et al. (2002) found that DES markedly induced vitellogenin (VTG) in juvenile fathead minnows (*Pimephales promelas*) at water concentrations $\geq$ 300 ng/L, the lowest dose tested. Similarly, Folmar et al. (2002) reported that DES induced VTG in male sheepshead minnows at the lowest concentration they tested, 20 ng/L. Zhong et al. (2005) employed a full life-cycle test with the Chinese rare minnow using water concentrations of DES ranging from 50 to 5000 ng/L. Significant impacts both on estrogen-responsive and apical reproductive endpoints were observed at all test concentrations. These different *in vivo* studies suggest that DES may be quite potent in fish. This is

consistent with recent *in vitro* work by Lange et al. (2012) with cloned ERs from a variety of fish species, including the fathead minnow, suggesting that the potency of DES may be comparable to, or even exceed that of 17α-ethinylestradiol (EE2), a synthetic estrogen with a predicted no-effect concentration in fish lower than 1 ng/L (Caldwell et al., 2008).

3.2. Advantages of Biomarkers

- Biomarkers are effective early warning signals of adverse biological effects.
- Biomarkers can indicate biological effects, while chemistry-based surveillance system cannot.
- Biomarkers are more effective in revealing overall toxicities of complex mixtures.
- Biomarkers are economical.
- In recent years, the impact of aquatic pollution on human and animal life has become a matter of great concern. Fish responses have been used as biomarkers of aquatic pollution. The use of a suitable biomarker with different degrees of specificity is an important aspect of environmental monitoring based on biomarkers (Sarkar et al., 2006). In this direction an effort has been made to develop the biomarker responses as an early warning signal in pollution assessment.

3.3. Importance of biomarkers in monitoring aquatic and environmental pollution

In order to assess the impact of neurotoxic compounds on marine environment, the evaluation of acetylcholinesterase activity in marine organisms was used as a biomarker of exposure to neurotoxic agents such as organophosphorus, carbamate pesticides etc (Sarkar et al., 2006). Metallothioneins (MTs) are induced by toxic metals such as Cd, Hg, and Cu by chelation through cysteine residues and are used in both vertebrates and invertebrates as a biomarker of metal exposure (Sarkar et al., 2006). The measurement of the levels of DNA integrity in marine organisms such as Sea stars (*Asterias rubens*) from the North Sea and the marine snails (*Planaxis sulcatus*) from the Arabian Sea along the Goa coast exposed to environmental xenobiotic contaminants in a study by Sarkar et al. (2006) clearly indicated the extent and the nature of pollution at the sampling sites along coastal environment.

Knowledge of acute toxicity of a xenobiotic often can be very helpful in predicting and preventing acute damage to aquatic life in receiving waters as well as in regulating toxic waste discharges. A perusal of the available literature reveals that studies on the acute effects of toxic metals on the biochemical constituents of fishes are scanty (El-Naga *et al*., 2005; Azmat et al., 2008).

The traditional approach to assessing sediment quality involves chemical analysis. However, this is not sufficient to ensure safe disposal of dredged material or to determine the impact on biota of sediment contamination (Martín-Díaz et al., 2004). The toxic effect on flora and fauna is related to the bioavailability of contaminants. Because of the potential transfer of contaminants along food chains, not only the local habitat can be affected, but also organisms at higher trophic levels. The consumption of seafood may lead to adverse effects on human health (Martín-Díaz et al., 2004).

Biomarkers are useful tools as early warnings to determine exposure to contaminants and the effect of contaminants on organisms before the damage becomes irreversible (Martín-Díaz et al., 2004). They may link the bioavailability of compounds with their concentrations at target organs and intrinsic toxicity. Nevertheless, other confounding factors must be taken into account when the results are interpreted (Martín-Díaz et al., 2004).

We review different biomarkers used to determine the quality of marine sediment and dredged material. Also, we consider evaluation of the use of biomarkers in environmental risk assessment (ERA) and links between laboratory and field surveys. We integrate available information to determine the validity of the different biomarkers, their relevance for assessing contaminated sediments and the suitability of the methodology. We consider the results in a tiered approach to testing at levels characterizing toxic effects and validating *in situ* changes (Martín-Díaz et al., 2004).

The systematic development and application of biomarkers in environmental health risk assessment is a relatively new field (Vainio, 2001). At first, the major interest was in biomarkers of exposure, borrowing concepts from pharmacology, then it moved from the external estimates of exposure to internal measures of dose, and ultimately, to markers of target dose. While these markers provide evidence of exposures, they do not provide evidence of that toxicological damage has occurred (Vainio, 2001). For this reason, measurements of DNA adducts and protein adducts are of interest, since they may provide bridges between exposures and disease end-points. In parallel, more quantitative and more sensitive end-points for diseases have been sought (Vainio, 2001). Again, with advancing techniques in cytogenetics, extensive studies were conducted on such markers as chromosomal aberrations, micronuclei and other changes deemed to represent genomic damage (Vainio, 2001). However, these types of end-points

are quite unspecific for application to new hazards of uncertain human toxic (carcinogenic) potential (Vainio, 2001).

The efficiency of EDC testing programs could be enhanced through the use of emerging technologies in the areas of genomics and computational biology to provide mechanistic insights as to exposures and possible adverse effects in animals, such as fish (e.g., Ankley et al., 2006; Hook et al., 2006; Samuelsson et al., 2006; Hoffmann et al., 2006, 2008; Filby et al., 2007; Martyniuk et al., 2007; Ankley et al., 2009). This type of approach is consistent with recent recommendations from the National Research Council (NRC, 2007), who suggest a shift toward greater use of short-term (e.g., in vitro) assays and predictive toxicology tools for assessment of human health risks of chemicals. These approaches, which could encompass techniques ranging from computational models to in vitro assays and short-term in vivo tests, would help provide regulatory agencies throughout the world with cost-effective, predictive tools for monitoring and testing EDCs (Ankley et al., 2009).

3.4. Use of biomarkers in risk assessment

The systematic development and application of biomarkers in environmental health risk assessment is a relatively new field (Vainio, 2001). At first, the major interest was in biomarkers of exposure, borrowing concepts from pharmacology, then it moved from the external estimates of exposure to internal measures of dose, and ultimately, to markers of target dose. While these markers provide evidence of exposures, they do not provide evidence of that toxicological damage has occurred (Vainio, 2001). For this reason, measurements of DNA adducts and protein adducts are of interest, since they may provide bridges between exposures and disease end-points. In parallel, more quantitative and more sensitive end-points for diseases have been sought (Vainio, 2001). Again, with advancing techniques in cytogenetics, extensive studies were conducted on such markers as chromosomal aberrations, micronuclei and other changes deemed to represent genomic damage (Vainio, 2001). However, these types of end-points are quite unspecific for application to new hazards of uncertain human toxic (carcinogenic) potential (Vainio, 2001).

Experimental studies showed that flounder fish acquire contaminants with the food rather than by passive uptake via skin or gills (Mondon et al. 2001; Skouras et al., 2003). Broeg et al. (1999) showed that the stability of hepatocyte lysosomes was modulated in a delicate manner. Thus the integrity of hepatocyte lysosomes provided valuable information for the interpretation of the expression of cytochrome P450 1A in liver cells of the same individual (Skouras et al., 2003). The results presented by Skouras et al. (2003) underlined that biological effects of environmental contaminants can be monitored by means of immunological assays in the 'field'.

The present study was part of an integrated monitoring programme on flounder, which has showed that in conjunction with other physiological data from the same individual, innate immune parameters also allowed the observation of pollution effects. Cellular function such as uptake of neutral red was impaired in individuals with increased proportions of DNA adducts or decreased stability of lysosomes.

Also in the study by Skouras et al. (2003), the activity of plasma lysozyme was also decreased in individuals with impaired lysosome stability, and showed some correlation to cytochrome P450 1A induction. This underlines that innate immune parameters such as plasma lysozyme activity or phagocyte functions form valuable parameters as parts of an integrated monitoring programme (Skouras et al., 2003).

3.5. Biomarkers as tools to assess sediment quality: Laboratory and field surveys

The traditional approach to assessing sediment quality involves chemical analysis (Martín-Díaz et al., 2004). However, this is not sufficient to ensure safe disposal of dredged material or to determine the impact on biota of sediment contamination. The toxic effect on flora and fauna is related to the bioavailability of contaminants. Because of the potential transfer of contaminants along food chains, not only the local habitat can be affected, but also organisms at higher trophic levels. The consumption of seafood may lead to adverse effects on human health (Martín-Díaz et al., 2004).

Biomarkers are useful tools as early warnings to determine exposure to contaminants and the effect of contaminants on organisms before the damage becomes irreversible (Martín-Díaz et al., 2004). They may link the bioavailability of compounds with their concentrations at target organs and intrinsic toxicity. Nevertheless, other confounding factors must be taken into account when the results are interpreted (Martín-Díaz et al., 2004). Different biomarkers have been used to determine the quality of marine sediment and dredged material. Also, the use of biomarkers in environmental risk assessment (ERA) has been evaluated and linked between laboratory and field surveys. Available information has also been integrated to determine the validity of the different biomarkers, their relevance for assessing contaminated sediments and the suitability of the methodology

(Martín-Díaz et al., 2004). In a report by Martín-Díaz et al. (2004) results has been considered in a tiered approach to testing at levels characterizing toxic effects and validating *in situ* changes.

3.7. Innate immune responses as useful parameters to monitor cellular functions in a battery of biomarkers

Immunological biomarkers that reflect the effects of exposure to environmental contaminants in coastal marine habitats were sought and studied by Skouras et al. (2003) in European flounder (*Platichthys flesus*) from five locations in the German Bight with different anthropogenic impacts. Some general trends was drawn: plasma lysozyme activity was decreased in flounder contaminated with DDT adducts and some PCBs, while cellular functions such as phagocytosis and respiratory burst were stimulated by some chlorinated hydrocarbons (Skouras et al., 2003).

In the Skouras et al. (2003), measurements of innate immune responses of fish was implemented in an integrated biological effect monitoring programme on European flounder (Platichthys flesus) in the North Sea. The flounder is widely distributed in different habitats of the North and Baltic Seas. Like other marine flatfishes, it lives in close contact with the sediment and feeds on various benthic organisms (Skouras et al., 2003). Thus, marine flatfish species are frequently used as sentinel species in international monitoring programmes of biological effects of contaminants in coastal waters and estuaries (ICES 1996; 1999; Skouras et al., 2003).

The data reported by Skouras et al. (2003) were part of a monitoring programme which was conducted on flounder collected at several locations in the German Bight and which included the analysis of biochemical, pathological and parasitological parameters of the same individual as well as the measurement of some innate immune responses.

When considering heavy metal and chlorinated hydrocarbon contaminations in flounder muscle, Skouras et al. (2003) reported very low residues in animals from all the sites in their study, and there was no 'clean' site with all the residues 'below detection limit' (Dizer et al. 2003). In this situation, when analysing immunological data in their response to pollution, clear differences between sampling locations which could be confirmed in all the sampling campaigns could not be established (Skouras et al., 2003). According to Skouras et al. (2003), this was mainly an effect of the high variation in plasma lysozyme level, as well as phagocyte activity, at some of the locations.

Correlation analysis also revealed connections between the parameters applied by Skouras et al. (2003) and some contaminants as well as with some biochemical parameters used as biomarkers in pollution monitoring. In flounder with decreased integrity of hepatocyte lysosomal membranes, immune functions were impaired, and plasma lysozyme as well as phagocytosis activity of head kidney cells were impaired when the activity of cytochrome P450 1A was induced (Skouras et al., 2003). The data presented by Skouras et al. (2003) in their study indicated that innate immune responses may be useful parameters to monitor cellular functions in a battery of biomarkers of different levels of biological organisation.

4. Applications and Limitations of biomarkers in monitoring aquatic and environmental pollution

The applications, advantages and limitations of diagnostic and prognostic tests using biomarkers have been discussed by some authors such as Livingstone (1993). There are established and developmental biomarkers in mussels responsive to exposure to organic contaminants, including some indicative of damage to DNA ("comet" assay) putative induction of biotransformation enzymes (CYP1A-like protein), contaminant removal (MXR-like protein), lysosomal membrane damage and impairment of membrane function (Livingstone et al., 2000).

Biochemical markers have been selected among early molecular events occurring in the toxicological mechanisms of main contaminants (Banni et al., 2005). In the past 25 years, numerous biomarkers have been developed with the objective to apply them for environmental biomonitoring (Sanchez and Porcher, 2009). Recently, the Water Framework Directive (WFD) of the European Union specified monitoring programs required to assess the achievement of good chemical and ecological status for all water bodies by 2015 (Sanchez and Porcher, 2009). These biomarkers are being used to evaluate exposure of various species of sentinel marine organisms (e.g. mussels, clams, oysters, snails, fishes, etc.) to and the effect of various contaminants (organic xenobiotics and metals) using different molecular approaches [biochemical assays, enzyme linked immuno-sorbent assays (ELISA), spectrophotometric, fluorometric measurement, differential pulsed polarography, liquid chromatography, atomic absorption spectrometry].

Exposure to and toxic effects of contaminants can be measured in terms of the biochemical responses of the organisms-so-called molecular biomarkers (Livingstone, 1993). The hepatic biotransformation enzyme cytochrome P4501A in fish and other vertebrates is specifically induced by

organic contaminants such as aromatic hydrocarbons, PCBs and dioxins, and is used as a biomarker of exposure to organic pollution (Livingstone, 1993). Its induction is involved in chemical carcinogenesis via catalysis of the covalent binding of organic contaminants to DNA (DNA-adducts).

Azmat et al. (2008) attempted to investigate bioaccumulation of heavy metals in marine and fresh water fishes and their acute effects on some biochemical profiles to show an important link in the aquatic food chain. Biochemical profiles in fish and other aquatic organisms under heavy metal stress serve as important bioindicators in the monitoring of aquatic environment. On the basis of the Azmat et al. (2008) investigation, it may be concluded that concentration of heavy metals in fish of Sindh region is a matter of serious fact because ultimately its accumulate in human body and can cause damages in human body therefore heavy metals in the tissues of aquatic animals should occasionally monitored. As the heavy metal concentration in tissues reflects past exposure via water or food. It can demonstrate the current situation of the animals before toxicity affects the ecological balance of population in the aquatic environment (Azmat et al., 2008).

4.1. Studies on the marine environment quality

Azmat et al. (2008) also suggested that Pakistan coastal metal management smelting facility shell required assessing the potential toxicity of metals-contaminated effluent at its point of discharge to avoid the determining effects of toxic metals in high quality food. Otherwise changes in fish health due to pollution may decline in fish population. In a study on the marine environment quality along the Tunisian coasts using a statistical approach, Clams (*Ruditapes decussatus*) were collected by Banni et al. (2005) during the four seasons of 2003 on seven different sites from the Tunisian coasts. Oxidative stress was evaluated by Banni et al. (2005) in gills using catalase activity (Cat), neutral lipids and malonedialdehyde accumulation. Their study showed that glutathione *S*-transferase activity is related to the conjugation of organic compounds and was evaluated in both, gills and digestive glands (Banni et al., 2005). The study also evaluated acetylcholinesterase activity as the biomarker of exposure to organophosphorous, carbamate pesticides and heavy metals. For each biomarker, a discriminatory factor was calculated and a response index allocated and Banni et al. (2005) showed that samples from Gargour had the highest Multimarker Pollution Index during the four seasons, indicating higher contamination level.

Use of protein carbonyl has been well documented in humans and rodents. An attempt has also been made to study protein carbonyl as a biomarker of exposure in fish (Sarkar et al., 2006). Assay of carbonyl groups in proteins by provides a conventional technique for detecting and quantifying oxidative modification of proteins. A study was undertaken by Sarkar et al. (2006) to investigate the modulatory role of copper on non-enzymatic antioxidants viz., protein and non-protein thiols, ascorbic acid and metallothionein. Sarkar et al. (2006) also investigated possible regulatory role of copper on iron in fish.

Azmat et al. (2008) also estimated the heavy metal pollution in marine and fresh water and their acute toxicity and its toxicological affects on survival, physiological and biochemical parameters of the widely consumed fresh water and marine water fishes of Sindh. According to their study (Azmat et al., 2008), four bioindicators, two from marine water (*Liza subviridus* and *Johnius belengerii*) and two from fresh water species (*Cyprius carpio* and *Pomodasy argyrew*) were collected to study the species-site interaction. Water samples from both stations were also collected to analyze the essential and non essential metals and muscles of fish for metabolic parameters and persistent metals pollutant (Azmat et al., 2008). Total lipids, proteins, amino acids and glycogen were estimated by Spectrophotometery whereas Atomic Absorption Spectrophotometery was used for metals detection. The results of the Azmat et al. (2008) study showed that interaction of metal pollutants vary specie to specie. This showed that pollutants act by changing the structural or biological function of bioindicator. High concentrations of contaminants were found in tissues of fishes collected from marine water as compared to fresh water fishes (Azmat et al., 2008).

According to the study by Azmat et al. (2008), untreated wastes of industrial, technological and agricultural origin containing various metallic compounds often contaminate natural waters. Heavy metals due to their bio-accumulative and non-biodegradable properties constitute a core group of aquatic pollutants (Azmat et al., 2008). These metals particulates enter the aquatic medium through effluents discharged from tanneries, textiles, electroplating, metal finishing, mining, dyeing and printing industries, ceramic and pharmaceutical industries etc. (Azmat and Talat, 2006). They concentrate in the tissues of aquatic biota and are known to produce cumulative deleterious effects (Cosson, 1994).

4.2. Biotechnology, aquatic and environmental pollution monitoring

Metals and organic contaminants, present in the water-column, sediment or food, are readily accumulated by aquatic organisms (Livingstone, 1993). Exposure to and toxic effects of contaminants can be measured in terms of the biochemical responses of the organisms-so-called molecular biomarkers (Livingstone, 1993). The applications, advantages and limitations of such diagnostic and prognostic tests are discussed. The hepatic biotransformation enzyme cytochrome P4501A in fish and other vertebrates is specifically induced by organic contaminants such as aromatic hydrocarbons, PCBs and dioxins, and is used as a biomarker of exposure to organic pollution (Livingstone, 1993). Its induction is involved in chemical carcinogenesis via catalysis of the covalent binding of organic contaminants to DNA (DNA-adducts) (Livingstone, 1993).

4.3. Studies on the application of biomarkers in monitoring aquatic pollution using Bioindicators

The toxicity tests are necessary in water pollution evolution because chemical and physical measurements alone are not sufficient to assess potential effects on aquatic biota (Azmat et al., 2008). Analysis of metals in water samples from marine and fresh water resources by Azmat et al. (2008) showed that there is a significant difference in concentration of pollutants that were higher in marine water as compared to fresh water (Azmat et al., 2008). In addition, it is an important step to detect the level of toxicants and their effects in the marine organism (Azmat et al., 2008). Such effects might lead to integrated effects on metabolic functions such as behavioral, growth, reproduction and survival. This can result in changes in fish health and reproduction that may alter fish population and community structure (Azmat et al., 2008).

The study by Azmat et al. (2008) also showed that although there is significant difference in concentration in of metallic ions in both aquatic resource but rate of accumulation of these elements were same as the Na, K, Ca and Mg are very important minerals elements and found insoluble salts in the sacroplasm of the muscular cells, inter cellular fluid, blood and plasma (Azmat *et al*., 2006). The values of macronutrients reported by Azmat et al. (2008) showed decline in concentration compared with literature which may affects fish health because these elements also play an important role in physiological processes involves in structure of several organic compounds (Azmat et al., 2008). An increase in concentration of K, Na or Mg contents in sea-water may alter the morpho-functional changes in fishes (Azmat et al., 2008). These changes include the increase in the height and the diameter of the micli of pinealcytes, the increase being followed by apocrynic secretion in the cells which may disturb the ionic balance of internal miles (Deane and Woo, 2005).

4.4. Studies on Bioaccumulation potential of heavy toxic metals in four animal species

Azmat et al. (2008) also studied bioaccumulation potential of heavy toxic metals in four species *Cyprius carpio*, *Pomodasy argyrew Liza subviridus* and *Johnius belengerii* and compared their findings with international literature. Pb concentration in muscles of two biomarkers from fresh water (*Cyprius carpio*) and *Pomodasy argyrew* and two from marine water (*Liza subviridus* and *Johnius belengerii*) showed significant difference. It indicated that interaction of heavy toxic metals with biomarkers vary specie to specie and more prominent in marine water fish. These interactions occur at the cellular and molecular level and are the abilities of Pb to displace Ca during specific physiological process. It is likely that Pb blocks Ca efflux from cells by substituting Ca in Ca^{++}/Na adenosine triphosphate (Simons, 1986). The findings of their study (Azmat et al., 2008) indicate that different species have various capabilities to accumulate and store water contaminates independent of their level in water. Same phenomena were observed by De la Torre *et al*. (2000). Hg also interact with the metal binding protein metalothionine (MT), a low molecular weight cytosolic protein protect the biological system by binding metal ions. Higher concentration of amino acids reported in these fish also support Hg amino acid interaction, which may control Hg toxicity (Azmat et al., 2008).

Cadmium derives its toxicological properties from its chemical similarity to Zn, an essential micronutrient for plants, animals and human. Cd as an ion affects on respiration and binders in exchange of gases (Gulfaraz and Ahmed, 2001). Cosson (1994) reported that Zn ions of metallothionine (MT) molecule were replaced by those of Cd when both metals were combined in the organism. This metal also showed affinity to protein SH group. This may be related with interesting pattern of interaction between metal and biochemical constitutes of these species like protein, amino acids glycogen and total lipids content of these biomarkers (Azmat et al., 2008). Results obtained from biochemical analysis of these common edible fish can give a useful indication for proper use of biochemical response as biomarkers in monitoring water born pollution by heavy metals (Azmat et al., 2008).

Investigation showed that appreciable decline in the biochemical profiles such as total glycogen, total lipids and total protein contents of the fish in presence

of toxins, results in decrease productivity of fish population. However, the decrease in protein content was significant in marine water fish. This study by Azmat et al. (2008) reflects the extent of the toxic effects of toxic metals and the metal induced cumulative deleterious effects at various functional levels in the widely consumed freshwater fish and marine fish. The toxicity of heavy metal caused the glucose level to decrease with increase of pollutants concentration and decrease the glycogen content in muscle as reported by Scott *et al.* (2006 cited in Azmat et al., 2008).

4.5. Multi-biomarker approach for freshwater wetland pollution monitoring

Artificial lakes such as dams where still waters are found, due to their specific configuration and water dynamics, are more prone to receive and accumulate anthropogenic discharges resulting from domestic, municipal and industrial effluents as well as agriculture runoff. Hence, organisms' synergistic/antagonistic responses to a mixture of contaminants are hardly interpreted and predicted exclusively by chemical analysis of those contaminants (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007). The use of biomarkers has been demonstrated to be a suitable alternative for monitoring and management of these aquatic ecosystems (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007).

Three sets of biomarkers concerning (i) genotoxicity, (ii) oxidative stress responses and (iii) endocrine alterations have been reportedly adopted. The presence of pro and/or genotoxic compounds was detected as expressed in the DNA integrity loss induced in different target tissues (gill, blood and liver), displaying a decreasing genotoxic gradient from the initial to the ending part of the water body. In a study by Maria et al. (2006), Ahmad et al. (2006), Teles et al. (2007), the activation of antioxidant enzymes as well as their inhibition provided a clear indication of pollution presence and environmental health degradation. All the organs revealed a similar resistance to peroxidative damage (LPO), suggesting that the antioxidants are more responsive biomarkers than LPO for short-term exposures (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007). Endocrine and metabolic biomarkers revealed increased plasma cortisol and glucose concentrations at all exposure sites, signalling the presence of stressors (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007). According to Teles et al. (2007), thyroid metabolism disruption was detected at the closest site to the main pollution source and in addition, the observed eel's plasma 17β-estradiol increase indicates water contamination by this particular steroid (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007).

Despite the complexity of fish overall responses to mixtures of contaminants, the biomarkers by Teles et al. (2007) were able to express site-specific responses, demonstrating their ability to detect the presence of a wide range of chemicals (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007).

4.6. Studies using non-enzymatic antioxidants of fish with special reference to biomarkers in monitoring of aquatic pollution

In recent years, the impact of aquatic pollution on human and animal life has become a matter of great concern. Fish responses have been used as biomarkers of aquatic pollution (Parvez and Raisuddin, 2003). The use of a suitable biomarker with different degrees of specificity is an important aspect of environmental monitoring based on biomarkers (Parvez and Raisuddin, 2003). In this direction an effort has been made to develop the biomarker responses as an early warning signal in pollution assessment (Parvez and Raisuddin, 2003). Parvez and Raisuddin (2003) conducted studies on the different sites of river Yamuna in order to assess the pollution profile. In their (Parvez and Raisuddin, 2003) study, the oxidative responses and the antioxidant potential of fish differed in relation to species, habitat and feeding behaviour. Parvez and Raisuddin (2003) reported that the levels of heavy metals and pesticides varied concentrations at different sites. The findings by Parvez and Raisuddin (2003) provide an insight for the assessment of non-enzymatic antioxidants at two different sites for their successful use as biomarkers.

4.7. Protein carbonyl as a biomarker of exposure in fish

An attempt was also made to study protein carbonyl as a biomarker of exposure in fish. Use of protein carbonyl has been well documented in humans and rodents (Parvez and Raisuddin, 2003). Not until 2003, no study has been reported in case of fish. The findings by Parvez and Raisuddin (2003) showed a biomarker approach using the protein carbonyl content in fish. A significant increase in protein carbonyls have been observed in all groups of fish exposed to different pesticides. Assay of carbonyl groups in proteins provides a conventional technique for detecting and quantifying oxidative modification of proteins (Parvez and Raisuddin, 2003).

4.8. The modulatory role of copper on non-enzymatic antioxidants

A study was undertaken by Parvez and Raisuddin (2003) to investigate the modulatory role of copper on non-enzymatic antioxidants viz., protein and non-protein thiols, ascorbic acid and metallothionein. It investigated possible regulatory role of copper on iron in fish. Their findings (Parvez and Raisuddin, 2003) provided a new insight into the multifarious role of low-level exposure to copper in fish. It seems to protect the fish from peroxidative damage by inducing both non-enzymatic antioxidants and possibly antioxidant enzymes by the induction of ceruloplasmin and metallothionein (Parvez and Raisuddin, 2003). Findings of this study by Parvez and Raisuddin (2003) will be helpful in pollution monitoring and identification of pollutant-sensitive and pollutant-resistant Indian fish species. It will enable us to predict the pollution profile of an aquatic habitat viable for fishery activities. It will also contribute to the development of a battery of mechanism-based biochemical assays that can be used to characterize the complex mixtures of chemicals in different potentially toxic environments and thus enhance our ability to assess the long-term risk of environmental contaminants to human health (Parvez and Raisuddin, 2003).

4.9. Biomonitoring aquatic and environmental pollution using feral eel (*Anguilla anguilla*)

Le Bras (1984) determined the amounts of catecholamine (dopamine, adrenaline, and noradrenaline) in the brain, heart, and plasma of the eel (*Anguilla anguilla* L.) during a 24-hr period and at three different times of year by using a radioenzymatic method. Seasonal variations of catecholamine average values were found to be different when considering catecholamine levels in the same tissue (one exception: heart levels in May) or the same amine in different tissues (Le Bras, 1984). Circadian rhythms of catecholamine levels were evident only in the brain; the maximum amount generally occurred during the light phase. No correlation could be found between the 24-hr variations in the different tissues. The most important variations were phased with the dark-light cycle but were also dependent on the annual cycle (Le Bras, 1984).

van der Oost et al. (1997) collected sediments and eel (*Anguilla anguilla*) samples from six Amsterdam freshwater sites with varying degrees of pollution in a large-scale field study. Bivariate correlation analysis, principal component analysis (PCA) and residual maximum likelihood analysis (REML) all revealed that the eel tissue levels of most PCB and OCP analyte groups were suitable to assess exposure to these contaminants, whereas PAH tissue levels were not (van der Oost et al., 1997). The phase I biotransformation enzymes in eel were found to be the most responsive to organic pollutants in the environment. Phase II enzymes and cofactors, as well as DNA adducts, were found to be less sensitive biomarkers, whereas the antioxidant enzymes and the serum transaminases did not show statistically significant correlations with pollutant levels. Similar results were obtained by means of the postulated bivariate correlation-significance index (CSI) and the multivariate PCA analysis in that same study by van der Oost et al. (1997).

Van der Oost et al. (1996, 1997) carried out a study to select a set of relevant biomarkers in feral eel for the biological assessment of inland water pollution. A suite of biochemical parameters in eel (hepatic biotransformation enzymes and cofactors, antioxidant enzymes, PAH metabolites, DNA adducts, serum transaminases) was measured in order to determine their response to xenobiotic compounds in the environment. The findings of their study and the main conclusions drawn from the trends found for the levels and activities of biochemical parameters in eel were the following: the phase I biotransformation enzymes in eel liver appeared to be the most sensitive to environmental xenobiotics. Cytochrome b_5 ($Cytb_5$), cytochrome P450 1A (CYP1A), ethoxyresurofin-*O*-deethylase (EROD) and EROD turnover (EROD/P450) in eel liver showed significant responses to contamination, and can therefore be used as biomarkers (Van Der Oost et al., 1995, 1996, 1997). Levels of a CYP3A-like protein were significantly elevated in eel from three moderately polluted sites, but since this protein was not induced in eel from the most polluted site its relevance as a biomarker remains unclear (Van Der Oost et al., 1995, 1996, 1997).

van der Oost et al. (1997) used discriminant analysis (DA) to classify the pollution status of the various sites and it appeared that the best discrimination between reference sites, moderately polluted sites and heavily polluted sites was obtained using DA on data of the nine most responsive biochemical markers. The importance of monitoring biota for the classification of the pollution status or environmental quality of freshwater sites was demonstrated in the study by van der Oost et al. (1997), since no clear discrimination between moderately and heavily polluted sites could be made using sediment pollutant levels only.

4.10. The significance and application of Molecular Biomarkers in aquatic and environmental pollution monitoring

A suite of biomarkers are being extensively used worldwide to assess the impact of highly persistent

pollutants such as polychlorinated biphenyls (PCB), polychlorinated dibenzo–dioxins (PCDD), polychlorinated dibenzo–furans (PCDF), polynuclear aromatic hydrocarbons (PAH), tributyltin (TBT) and other toxic metals on the marine ecosystem (Sarkar et al., 2006). The induction of the biotransformation enzyme, cytochrome P4501A in fishes (*Callionymus lyra, Limanda limanda, Serranus* sp., *Mullus barbatus*) and mussels (*Dreissena polymorpha*) by various xenobiotic contaminants such as PCBs, PAHs, PCDs is used as a biomarker of exposure to such organic pollutants (Sarkar et al., 2006). The induction of cytochrome P4501A is involved in chemical carcinogenesis through catalysis of the covalent bonding of organic contaminants to a DNA strand leading to formation of DNA adducts (Sarkar et al., 2006). The measurement of the levels of DNA integrity in marine organisms such as Sea stars (*Asterias rubens*) from the North Sea and the marine snails (*Planaxis sulcatus*) from the Arabian Sea along the Goa coast exposed to environmental xenobiotic contaminants clearly indicated the extent and the nature of pollution at the sampling sites along coastal environment (Sarkar et al., 2006).

5. Concepts and Key issues associated with the implementation of biomarkers in aquatic and environmental monitoring

Several key issues associated with the use of biomarkers, which could influence their effectiveness and usefulness has been previously discussed (Lam, 2009). First, there are few biomarkers that are specific enough to allow an identification of the precise nature of environmental stressors. Second, biomarker studies conducted at molecular or subcellular levels tend to be more repeatable and predictable, but their ability to predict significant biological effects is limited. In contrast, biomarkers at physiological, organismic or higher levels are usually more ecologically relevant, but slower to respond and more difficult to detect. Third, some organisms have the ability to repair damage induced by initial toxic insults and make adjustments to their biological responses, thus increasing the chance of false negatives. Therefore, the selection of an appropriate biomarker for use under specific ecological circumstances will be a compromise that is determined by the precise question(s) asked and cost-benefit considerations (Lam, 2009).

Biomarkers are promising tools for biomonitoring, both in the marine and freshwater environment (den Besten, 1998). It is however clear that much more information is needed about the exact relation between biomarker responses and the health and fitness of organisms, and even more so between biomarker responses and risks for the ecosystem. In order to address these questions, it is important to realise that the possibilities for the application of a biomarker depend on the concept that is chosen for environmental monitoring. The purpose of monitoring programs varies from simple screening to risk characterisation on the ecosystem level. This paper compares a number of these concepts with respect to how biomarkers can be used and with respect to the specific requirements for further implementation of biomarkers in environmental monitoring (den Besten, 1998).

Furthermore, there is an increased public awareness regarding pesticides, fertilizers, agricultural products and metals that might endanger our indigenous fish populations and aquatic ecosystems (Azmat et al., 2008). This is mainly because humans use these natural resources as food and water supplies, are therefore also exposed to produce polluting these resources (Evans *et al*., 2000; Azmat et al., 2008). Of particular concern is the exposure of bio-organisms to metal pollution, as it is known that metals act as mutagenic/genotoxic compounds, interfere with xenbiotic metabolic pathways and may also affect glycolysis, the Krebs cycle, oxidative phosphoorylation, protein, amino acid metabolism as well as carbohydrate and lipid metabolism (Drastichová *et al*., 2005; De la Torre *et al*., 2000; Azmat et al., 2008).

The monitoring of biological effects has recently become an integral component of environmental monitoring programmes as a supplement to the commonly used contaminant monitoring (Lam and Gray, 2003). Over the years, many biomarkers have been developed that are claimed to be efficient at providing an early warning of deleterious effects on biological systems and for estimating biological effects due to contaminants (Lam and Gray, 2003). Although biomarkers are potentially useful, they have a number of important limitations (Lam and Gray, 2003). Lam and Gray (2003) examined some of the key assumptions behind the theory and practice of use of biomarkers, and proposed a scheme, which may facilitate decisions by environmental managers as to how and when to use biomarkers in their monitoring programmes.

5.1. Biomonitoring of aquatic and environmental pollution using Small Animal Models

In recognition of the utility of fish as surrogate models for other vertebrates, as well as documentation of impacts of EDCs on this class of animals in the field, different testing approaches utilizing fish are being developed and validated for regulatory

programs for EDCs both nationally and internationally (Ankley et al., 2004).

In the United States, a 1996 congressional mandate directed the Environmental Protection Agency (EPA) to develop a formal screening and testing program for EDCs (EPA 1998; Ankley et al., 2004). Five test systems were recommended by an advisory committee for Tier 1 screening for the EPA program (EPA 1998; Ankley et al., 2004). Three systems use rats, one uses an amphibian (*Xenopus laevis*), and the other uses a small fish, the fathead minnow (*Pimephales promelas*), in a short-term (21-day) assay (EPA 1998; Ankley et al., 2004). Chemicals identified as possible EDCs in Tier 1 screening might then be subjected to more intensive Tier 2 tests, which could include full-life cycle or even multigenerational assays with a number of vertebrate species including fish such as the fathead minnow, Japanese medaka (*Oryzias latipes*), or sheepshead minnow (*Cyprinidon variegatus*) (Ankley et al., 2004). From an international perspective, the Organisation for Economic Cooperation and Development (OECD1) has formed a task group focused on developing internationally harmonized test methods for EDCs for both mammalian and nonmammalian species (Huet 2000; Ankley et al., 2004). A subcommittee within the task group is currently focusing specifically on fish tests (Ankley et al., 2004). Three small fish species (fathead minnow, medaka, and zebrafish [*Danio rerio*]) are being evaluated for screening (partial-life cycle assays) as well as more extensive (full-life cycle) testing of EDCs (OECD 1999, 2000, 2004; Ankley et al., 2004).

According to Ankley et al. (2004), both from ecological effects and species extrapolation perspectives, fish tests are an important component of EDC screening and testing programs. Based on progress in this area, it is clear that fish models will continue to play an important role both in research and regulation of EDCs (Ankley et al., 2004).

5.2. Biomonitoring of aquatic and environmental pollution using Diethylstilbesterol as a model estrogen in toxicologically-oriented studies with fish

Diethylstilbestrol (DES) is a synthetic estrogen that has been banned for use in humans, but still is employed in livestock and aquaculture operations in some parts of the world (Adedeji et al., 2012). Detectable concentrations of DES in effluent and surface waters have been reported to range from slightly below 1 to greater than 10 ng/L. Little is known, however, concerning the toxicological potency of DES in fish (Adedeji et al., 2012). Although currently banned for this application in many countries, DES still is used in some parts of the world for livestock production (Adedeji et al., 2012). For example, studies monitoring environmental estrogens in effluents and surface waters from different locations in Asia have found DES concentrations on the order of 0.1 to 10 ng/L and, in several instances, associated this occurrence with terrestrial livestock operations (Jin et al., 2008; Chen et al., 2009, 2010; Zhang et al., 2010; Lu et al., 2011; Wang et al., 2011; Zhou et al., 2012; Adedeji et al., 2012). In the study by Adedeji et al. (2012), sexually-mature fathead minnows (*Pimephales promelas*) of both sexes were exposed to 1, 10 or 100 ng DES/L water in a flow-through system. The study showed that DES causes a range of responses in fish at water concentrations comparable to those reported in the environment, and that *in vivo* potency of the estrogen is on par with that of the better-studied estrogenic contaminant 17α-ethinylestradiol.

The biological effects-concentrations observed for DES are easily on par with those associated with adverse impacts of EE2 on fish (Caldwell et al., 2008), a compound frequently referred to as the most potent known estrogenic environmental contaminant. Further, DES concentrations associated with effects in our short-term fathead minnow experiment overlap with those of DES reported in multiple effluent and surface water monitoring studies from parts of the world where the estrogen appears to be used for terrestrial livestock and/or aquaculture applications (Jin et al., 2008; Chen et al., 2009, 2010; Zhang et al., 2010; Lu et al., 2011; Wang et al., 2011; Zhou et al., 2012; Adedeji et al., 2012). This indicates the need for additional reproductive and developmental toxicology data for DES effects in fish at concentrations reflective of those in the environment to accurately determine potential ecological risk (Adedeji et al., 2012).

The paper by Ankley et al. (2009) provided an overview and illustrative results from a large, integrated project that assesses the effects of EDCs on two small fish models, the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*). The studies employ a combination of state-of-the-art genomic (transcriptomic, proteomic, metabolomic), bioinformatic and modeling approaches, in conjunction with whole animal testing, to develop response linkages across biological levels of organization (Ankley et al., 2009). This understanding forms the basis for predictive approaches for species, endpoint and chemical extrapolation. Although the study by Ankley et al. (2009) is focused specifically on EDCs in fish, it was believed that the basic conceptual approach has utility for systematically

assessing exposure and effects of chemicals with other MOA across a variety of biological systems (Ankley et al., 2009).

5.3. Biomonitoring of aquatic and environmental pollution using Small Fish Models in Identifying and Assessing Effects of Endocrine-disrupting Chemicals

Endocrine-disrupting chemicals (EDCs), particularly those that affect the hypothalamic-pituitary-gonadal (HPG) axis of vertebrates, have become a focus of regulatory screening and testing throughout the world (Ankley et al., 2004). Small fish species, principally the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*), are used as model organisms for several of these testing programs. Fish are appropriate models for testing EDCs, not only from the perspective of existing ecological impacts, but also in terms of species extrapolation (Ankley et al., 2004).

Since the early 1990s, an international effort has focused on identifying possible adverse effects of endocrine-disrupting chemicals (EDCs1) on reproduction and development in both humans and wildlife (Ankley et al. 2004). The hypothalamic-pituitary-gonadal (HPG1) axis, especially aspects of the system directly related to steroid hormones (estrogens, androgens), has been of particular concern (EPA 1998; Huet 2000; Ankley et al., 2004).

A number of chemicals with the potential to affect the hypothalamic-pituitary-gonadal (HPG) axis of animals enter aquatic systems through a variety of point and nonpoint source discharges (Ankley et al., 2004). Not surprisingly, therefore, some of the better documented examples of adverse effects of EDCs in the environment are for aquatic animals, particularly fish (Ankley and Giesy 1998; WHO 2002; Ankley et al., 2004).

The low exposure concentration to EDCs, longer time frame exposure is more environmentally relevant because 17α-ethynylestradiol (EE_2) concentrations range from 0.5 to 15 ng EE_2/L in the aquatic environment (Ying et al., 2008; Ribeiro et al., 2008), and aquatic animals may be exposed to the chemical throughout their lifetime (Li et al., 2011).

5.4. Biomonitoring of aquatic and environmental pollution using physiologically-based computational model

Physiologically-based computational model represents the hypothalamic-pituitary-gonadal axis in adult female FHM robustly (Li et al., 2011). The model is useful to estimate how estrogens (e.g., 17α-ethynylestradiol) or androgens (e.g., 17β-trenbolone) affect plasma concentrations of 17β-estradiol, testosterone and vitellogenin, which are important determinants of fecundity in fish (Li et al., 2011).

Endocrine disrupting chemicals (e.g., estrogens, androgens and their mimics) are known to affect reproduction in fish (Li et al., 2011). 17α-ethynylestradiol is a synthetic estrogen used in birth control pills. 17β-trenbolone is a relatively stable metabolite of trenbolone acetate, a synthetic androgen used as a growth promoter in livestock (Li et al., 2011). According to Li et al. (2011), both 17α-ethynylestradiol and 17β-trenbolone have been found in the aquatic environment and affect fish reproduction. Li et al. (2011) in their study developed a physiologically-based computational model for female fathead minnows (FHM, *Pimephales promelas*), a small fish species used in ecotoxicology, to simulate how estrogens (i.e., 17α-ethynylestradiol) or androgens (i.e., 17β-trenbolone) affect reproductive endpoints such as plasma concentrations of steroid hormones (e.g., 17β-estradiol and testosterone) and vitellogenin (a precursor to egg yolk proteins).

Li et al. (2011) using Markov Chain Monte Carlo simulations, the model were calibrated with data from unexposed, 17α-ethynylestradiol-exposed, and 17β-trenbolone-exposed FHMs. In their (Li et al., 2011) study, the model predictions agreed with the experimental data well. In their study, the model predicted reproductive endpoints from independent studies well. For more than 85% of the simulation results, the 95% CIs of model predictions encompassed the median of the experimental data. To further evaluate the model's predictive ability; more experimental data are needed, especially for the endpoints in FHMs exposed to a mixture of TB and EE_2.

The HPG axis computational model for male FHM was previously described by Watanabe et al. (2009). The model simulates time continuously, but it does not have a seasonal component. The model for male FMH published by Watanabe et al. (2009) had no androgen receptor (AR) component. Watanabe et al. (2009) did not also include the binding process of EE_2 to SBPs in blood. Teeguarden and Barton (2004) in their modelling work for male FHMs, the total concentration of SBPs was assumed to be 20 nmol/L based upon a measurement in human males. In a recent study, Shoemaker et al. (2010) developed a computational model to simulate more detailed biochemical reactions in the FHM steroidogenic pathway. Their model did not incorporate any AR-related signalling pathways (Li et al., 2011). As AR plays an essential role for androgen responses and subsequent regulation of steroidogenesis, the recent model reported by Li et al. (2011) advances the work

of Shoemaker et al. (2010) by simulating AR-related signalling pathways.

5.4.1. Important new features of Li et al. (2011) model

Important new features of this model include: (i) the simulation of AR in multiple tissue compartments (i.e., brain, liver, and gonad); (ii) AR binding and its effects upon the HPG axis; and (iii) free androgen effects on brain AR concentration. As a result, this model provides a computational framework for endocrine responses of EDCs functioning through both ER and AR.

The two EDCs, 17α-ethynylestradiol and 17β-trenbolone used in the Li et al. (2011) model, have been widely studied as model estrogens and androgens, respectively (Länge et al., 2001; Ankley et al., 2003; Orlando et al., 2004; Pawlowski et al., 2004; Seki et al., 2006). Both compounds also are environmentally relevant contaminants (Li et al., 2011).

According to Li et al. (2011), 17α-ethynylestradiol (EE_2), a synthetic estrogen used in birth control pills, enters the environment mainly through effluents from wastewater treatment facilities. The reported median EE_2 concentration in the aquatic environment varies from <0.5 to 15 ng/L (Ericson et al., 2002; Li et al., 2011). Due in part to its high binding affinity for estrogen receptor (ER) (Gale et al., 2004; Denny et al., 2005; Li et al., 2011), EE_2 affects the HPG axis in FHM at environmentally relevant concentrations. Exposure to EE_2 has been shown to result in altered hormone profiles, and increased vitellogenin (VTG, a precursor of egg yolk proteins) levels in both male and female FHMs (Parrott and Blunt, 2005; Li et al., 2011). In addition, a seven-year, whole-lake experiment conducted in Canada (Kidd et al., 2007) showed that chronic exposure of FHMs to 5 - 6 ng EE_2/L led to near-extinction of this species from the lake.

Also, the 17β-trenbolone (TB) is a relatively stable metabolic product of trenbolone acetate, a synthetic androgen used as a growth promoter in livestock (e.g., cattle) (Li et al., 2011). TB enters the environment mainly as runoff from livestock feedlots (Li et al., 2011). Previous studies have reported the use of 17β-trenbolone (TB) as model. Schiffer et al. (2001) reported that the TB concentration in effluents of solid cattle dung was around 19 ng/L. Durhan et al. (2006) studied a cattle feedlot located in southwest central Ohio, and reported that the TB concentration in feedlot discharge was between 10 and 20 ng/L. According to Li et al. (2011), TB has a high binding affinity for the androgen receptor (AR). Water exposure to TB at concentrations similar to those found in the environment decreases egg production in FHM in conjunction with changes in plasma concentrations of 17β-estradiol (E_2), testosterone (T), and VTG in females (Ankley et al., 2003; Li et al., 2011). Interestingly, relationships between TB water exposure concentrations and plasma E_2, T and VTG concentrations were not monotonic, but were "U-shaped" (Ankley et al., 2003; Li et al., 2011).

5.5. Biomonitoring of aquatic and environmental pollution using Expression signatures for a model androgen and antiandrogen

Certain endocrine-active toxicants have been reported to completely sex reverse both male and female individuals in amphibian, avian, fish, invertebrate, and reptile species, resulting in a phenotype indistinguishable from unaffected individuals (Olmstead et al., 2011). Detection of low-level sex reversal often requires large numbers of organisms to achieve the necessary statistical power, especially in those species with predominantly genetic sex determination and cryptic/homomorphic sex chromosomes (Olmstead et al., 2011). Here we describe a method for determining the genetic sex in the commonly used ecotoxicological model, the fathead minnow (Pimephales promelas). Analysis of amplified fragment length polymorphisms (AFLP) in a spawn of minnows resulted in detection of 10 sex-linked AFLPs, which were isolated and sequenced (Olmstead et al., 2011). No recombination events were observed with any sex-linked AFLP in the animals examined (n=112). A polymerase chain reaction (PCR) method was then developed that determined the presence of one of these sex-linked polymorphisms for utilization in routine toxicological testing (Olmstead et al., 2011). Analyses of additional spawns from our in-house culture indicate that fathead minnows utilize a XY sex determination strategy and confirm that these markers can be used to genotype sex; however, this method is currently limited to use in laboratory studies in which breeders possess a defined genetic makeup (Olmstead et al., 2011). The genotyping method described herein can be incorporated into endocrine toxicity assays that examine the effects of chemicals on gonad differentiation (Olmstead et al., 2011).

Trenbolone, an anabolic androgen, and flutamide, an antiandrogen, are prototypical model compounds for agonism and antagonism of the androgen receptor (Garcia-Reyero et al., 2009). We hypothesized that 48 h exposures of female fathead minnows (Pimephales promelas) to environmentally relevant concentrations of these chemicals would alter genes regulated by the androgen receptor and that a mixture of the two compounds would block the effects

(Garcia-Reyero et al., 2009). Gene expression in the ovaries was analyzed using a fathead minnow-specific 22,000-gene microarray. Flutamide altered abouttwicethe number of genes astrenbolone, most of which appeared to be through pathways not associated with the androgen receptor (Garcia-Reyero et al., 2009). A group of 70 genes, of which we could identify 37, were reciprocally regulated by trenbolone and flutamide. These are candidates for specific biomarkers for androgen receptor mediated gene expression (Garcia-Reyero et al., 2009). Four genes stand out as specifically related to reproduction: sperm associated antigen 8 (SPAG8), CASP8 and FADD-like apoptosis regulator (CFLAR), corticotropin releasing hormone (CRH), and 3beta-hydroxysteroid dehydrogenases (3beta-HSD) (Garcia-Reyero et al., 2009). Three notable transcriptional regulators including myelocytomatosis viral oncogene homologue (MYC), Yin Yang 1 (YY1), and interferon regulator factor 1 (IRF1) may function as early molecular switches to control phenotypic changes in ovary tissue architecture and function in response to androgen or antiandrogen exposure (Garcia-Reyero et al., 2009).

6. Future trends in the use of biomarkers in monitoring aquatic pollution

The results obtained in a study by Sarkar et al. (2006) provided an insight for the assessment of non-enzymatic antioxidants at two different sites for their successful use as biomarkers. It also provided a new insight into the multifarious role of low-level exposure to copper in fish. It seems to protect the fish from peroxidative damage by inducing both non-enzymatic antioxidants and possibly antioxidant enzymes by the induction of ceruloplasmin and metallothionein. The findings of Sarkar et al. (2006) will be helpful in pollution monitoring and identification of pollutant-sensitive and pollutant-resistant Indian fish species. It will enable researchers and scientists to predict the pollution profile of an aquatic habitat viable for fishery activities. It will also contribute to the development of a battery of mechanism-based biochemical assays that can be used to characterize the complex mixtures of chemicals in different potentially toxic environments and thus enhance our ability to assess the long-term risk of environmental contaminants to human health.

To manage effectively aquatic resources and their habitats in the future will require an ability to relate results from laboratory research and field experiments to data from monitoring of contaminants and effects on fisheries and other resources (Pearce and Despres-Patanjo, 1988). Several preliminary strategies have evolved which permit scientists to measure, through time, change in contaminant loading, fates of contaminants as these pass into estuarine and marine ecosystems, and consequences as contaminants enter biological systems. The most pressing need now is to develop an interagency consensus in regard to standard procedures and criteria whereby habitat change can be measured, effects assessed, and actions taken to stop or reverse aquatic habitat degradation in coastal waters (Pearce and Despres-Patanjo, 1988).

A. anguilla in situ trial proved its high ability for freshwater monitoring, contributing to a better knowledge of fish toxicological responses to mixtures of contaminants (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007). The monitoring efficacy can be greatly improved by using batteries of non-specific biomarkers comprising different biological levels (Maria et al., 2006; Ahmad et al., 2006; Teles et al., 2007).

The results of the study by van der Oost et al. (1997) indicated that biological effect monitoring is the only appropriate method providing a reliable environmental risk assessment. Recent work focusing on more specific early-effect markers such as certain oncogenes and tumour-suppressor genes have substantial promise as shown by work with aflatoxins and vinyl chloride (Vainio, 2001). Such studies have also enhanced mechanistic insight (Vainio, 2001). The advances in molecular genetics have led to an upsurge in interest in most susceptibility factors, and identification of polymorphisms of various enzymes has become possible (Vainio, 2001). Ongoing search for “ultra-high risk” individuals may be fruitful, but probably only relevant to a small segment of potentially exposed populations (Vainio, 2001). Factors associated with a small differential risk, however theoretically or mechanistically important, offer only little practical use (Vainio, 2001).

Knowledge of possible toxic mechanisms (or modes) of action (MOA) of chemicals can provide valuable insights as to appropriate methods for assessing exposure and effects, thereby reducing uncertainties related to extrapolation across species, endpoints and chemical structure (Ankley et al., 2009). However, MOA-based testing seldom has been used for assessing the ecological risk of chemicals. This is in part because past regulatory mandates have focused more on adverse effects of chemicals (reductions in survival, growth or reproduction) than the pathways through which these effects are elicited (Ankley et al., 2009). A recent departure from this involves endocrine-disrupting chemicals (EDCs), where there is a need to understand both MOA and adverse outcomes (Ankley et al., 2009). To achieve this understanding, advances in predictive approaches

are required whereby mechanistic changes caused by chemicals at the molecular level can be translated into apical responses meaningful to ecological risk assessment (Ankley et al., 2009).

A prior knowledge of MOA can lead to identification of mechanism based (and, hence, stressor-specific) molecular indicators that can potentially be linked to environmental concentrations and used to inform exposure assessments (Ankley et al., 2009). Furthermore, knowledge of MOA can serve as a basis for effective extrapolation of biological effects across species, biological levels of organization, and chemical structures (Ankley et al., 2009). This information can help identify potentially sensitive responses, and even species prior to extensive testing, thereby optimizing time and resource use (Bradbury et al., 2004; Ankley et al., 2009).

We feel that the research approach presented by Ankley et al. (2009) provided a broad conceptual framework for developing mechanism-based, predictive approaches for effectively assessing the ecological risk of chemicals with a variety MOA, in addition to EDCs.

The model described by Li et al. (2011) can be used to generate hypotheses to facilitate studies of endocrine responses in female FHMs exposed to other estrogenic EDCs in addition to EE_2, or other androgenic EDCs in addition to TB. According to Li et al. (2011), the application of the model can be achieved by defining chemical-specific parameters, such as partition coefficients (e.g., blood to water, or tissue to blood), and binding affinities to ER and AR. Furthermore, the endpoints simulated in their (Li et al., 2011) study (i.e. plasma E_2, T and VTG concentrations) are important determinants affecting egg production in FHMs. In the future, this model could be linked to an oocyte growth dynamics model developed by Li et al. (accepted). According to Li et al. (2011), linking these two models would build a connection between EDC effects at a molecular level with effects upon an organism, and thus a population, which is an urgent need in ecological risk assessment.

7. Conclusion

On the basis of most studied reviewed, it may be concluded that concentration of heavy metals in aquatic environments is a matter of serious fact because ultimately its accumulate in human body and can cause damages in human body therefore heavy metals in the tissues of aquatic animals should be occasionally monitored. As the heavy metal concentration in tissues reflects past exposure via water or food. It can demonstrate the current situation of the animals before toxicity affects the ecological balance of population in the aquatic environment. Therefore, it is suggested that most aquatic and environmental management authorities required assessing the potential toxicity of metals-contaminated effluent at its point of discharge to avoid the determining effects of toxic metals in high quality food. Otherwise changes in fish health due to pollution may decline in fish population. However, the use of molecular, biochemical and cellular biomarkers has proved to be very useful in environmental and aquatic pollution monitoring.

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