Proceedings of Basic and Applied Sciences
ISSN 1857-8179 (paper). ISSN 1857-8187 (online). http://www.anglisticum.mk
Proceedings of the 1st International Conference on New Horizons in Basic and Applied Science, Hurghada – Egypt, Vol 1(1), 2013.

Biochemical and Physiological Biomarkers in Aquatic Environmental Research

Alaa G. M. Osman
Department of Zoology, Faculty of Science
Al-Azhar University (Assiut Branch), 71524 Assiut, Egypt. E-mail: agosman2@yahoo.com

Received November 13, 2013; Revised November 14, 2013; Accepted November 15, 2013

Abstract: *Biomarkers are used as tools to assess biological changes that may reveal exposure of organisms to environmental chemicals. In some cases, biomarkers are able to indicate that chemicals specifically affect metabolic pathways or physiological functions in exposed individuals. Therefore, biomarkers can be used as both diagnostic and predictive tools. More recently, the concept of "biomarkers" has gained popularity amongst environmental managers. There are many different biomarkers that occur at many different levels of organization from sub-cellular to whole-organisms. Biomarkers at the molecular level tend to respond first, followed by responses at the cellular (biochemical and physiological), and whole-body levels. within this review, I will review the application of some biochemical and physiological biomarkers in aquatic environmental research. This review presents a synthesis of the state of the art in the methodology of biochemical and physiological biomarkers and its contribution in aquatic environmental research. The text explores the latest knowledge and thinking on this very important approach for the assessment of environmental health, management, and conservation. The primary concern of the present review is the measurement of biomarkers in aquatic organisms under field and laboratory conditions, where effects of chemicals at different levels of biological organization can be examined.*

Keywords: *Biomarkers, aquatic organisms, biochemical biomarkers, physiological biomarkers*

Introduction

Biomarkers are used as tools to assess biological changes that may reveal exposure of organisms to environmental chemicals. In some cases, biomarkers are able to indicate that chemicals specifically affect metabolic pathways or physiological functions in exposed individuals. Therefore, biomarkers can be used as both diagnostic and predictive tools. A number of case-studies demonstrated that biomarkers are useful tools to detect the presence of xenobiotics in living organisms and to diagnose individual health. Biomarkers have been proposed as sensitive tools for detecting environmental exposure and adverse effects of toxic anthropogenic chemicals on aquatic organisms. More recently, the concept of "biomarkers" has gained popularity amongst environmental managers. There are many different biomarkers that occur at many different levels of organization from sub-cellular to whole-organisms (Connell et al., 1999). Biomarkers at the molecular level tend to respond first, followed by responses at the cellular (biochemical and physiological), morphological/histological and whole-body levels. Thus, by monitoring molecular, biochemical and physiological parameters biomarkers can be used as an early warning system and the potential harm of an agent can be assessed before more severe disturbances/consequences occur (Lam & Gray, 2003). The biomarkers of early warning system are very sensitive to environmental stressors and rapidly-responding biomarkers but they are generally characterized by low ecological significance. Previously, I have reviewed the application of the molecular biomarkers in the aquatic environmental research (submitted). As a consequences, within this review, I will review the application of some biochemical and physiological biomarkers in aquatic

environmental research. This review presents a synthesis of the state of the art in the methodology of biochemical and physiological biomarkers and its contribution in environmental risk assessment. The text explores the latest knowledge and thinking on this very important approach for the assessment of environmental health, management, and conservation. The primary concern of the present review is the measurement of biomarkers in aquatic organisms under field and laboratory conditions, where effects of chemicals at different levels of biological organization can be examined.

Biochemical biomarkers

Biochemical indicators could be used (as biomarkers) to identify possible environmental contaminations before the health of aquatic organisms is seriously affected (Barnhoorn & Van Vuren, 2004 ; Jimenez & Stegeman, 1990) and to develop water quality indices (Gayet et al., 1993; Melancon, 1995; Powers, 1989; Zollner, 1993). Such biochemical approaches are among the first measurable responses to the presence of chemical pollutants and thus are early, sensitive indicators of possible damage at higher levels of organization, both in the organisms and possibly in the fish community (Casillas et al., 1983). There are many biochemical biomarkers and it is impossible and undesirable to cover them all here. In this review, two key examples which have proved popular in environmental assessment of the hazards of toxic chemicals to aquatic organisms are presented; (1) Alteration of enzymes and (2) structural changes in stress proteins.

Alteration of enzymes activity

In toxicological studies of acute exposure, changes in concentrations and activities of some enzymes may reflect cell damage in specific organs (Casillas, et al., 1983; Heath, 1996). Changes of maximum enzyme activities altered demands for the related metabolic function (Kiessling et al., 1991; Moon & Mommsen, 1987). This methodological approach has been used in numerous studies to investigate the influence of environmental factors on fish metabolism (Cowey et al., 1981; Hilton & Atkinson, 1982; Pelletier et al., 1994; Segner & Verreth, 1995).

The commonly used enzymes as biochemical biomarkers are Cytochrome P450 monooxygenases, glutathione S-transferases, Acetylcholinesterase, Glucose-6-Phosphate dehydrogenase and Lactate dehydrogenase.

In recent years, there has been a rapid development of enzymatic biomarkers. This is due not only to advances in biochemistry but also to modern methods of measurement (Lam & Gray, 2003). Such biomarkers generally include the deployment of detoxifying enzymes and antioxidant systems (Fitzpatrick et al., 1997; Livingstone et al., 1995). The suitability of various antioxidant parameters, such as glutathione S transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), glutathione (GSH) and lipid peroxidation, for use as biomarkers has been examined in a variety of aquatic organisms. For example, (Osman, 2012; Osman et al., 2010) studied the alteration in the activity of G6PDH and LDH in the tissues of African catfish and Nile tilapia collected from the whole course of the river Nile. These alterations go in parallel with the elevation in the levels of water chemical parameters detected in the water of Damietta and Rosetta sites as a result of pollution stress in these areas.

They concluded that, altered activities of G6PDH and LDH can provide a useful biomarker for environmental managers in investigating the exposure of fish to contaminated waters. In the same context, Sahan et al. (2010) analyzed the levels of pollution in the Ceyhan River (Turkey), subjected to agricultural and industrial pollution, and the effects of these pollutants on the gill and liver tissues of Cyprinus carpio. The biomarkers examined in the liver and gill tissues of the carp were superoxide dismutase (SOD), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PD) as well as glutathione (GSH) and lipid peroxidation (LPO). The activities of CAT, G6PD, GST and GSH were observed to be higher in the liver tissues of the fish in the polluted region. Tsangaris et al. (2010) measured a suite of biomarkers in caged mussels at areas impacted by different anthropogenic activities along the Greek coastline to assess biological effects of environmental pollution. Biomarkers indicative of neurotoxicity (acetylcholinesterase, AchE), oxidative stress (catalase, CAT), and phase II biotransformation of xenobiotics (glutathione S-transferase, GST), were measured to assess effects of various types of pollutants. AchE activity proved to be the most responsive biomarker with decreased values at sites influenced by agricultural, urban and industrial activities. Jemec et al. (2009) summarized their past experiences in biochemical biomarker research in two crustacean species. They assessed the intrinsic properties of biochemical biomarkers CAT, GST and ChE in the D. magna and the isopod P. scaber. They recommend that the use of biochemical markers is most appropriate for hazard identification because this is a procedure whose purpose is to characterize the potential hazard of the substance in question and is more flexible in terms of using different tools. They concluded that, the lesson learnt from biochemical biomarkers in environmental studies utilizing crustacean model species is that, for successful application of each group of biomarkers, their intrinsic properties are needed to be known before an (eco)toxicity study is designed. They suggest that a substantial body of experience obtained with biochemical biomarkers should be exploited to new emerging biomarkers in environmental studies in order to facilitate their application. As a part of a monitoring program Kurutas et al. (2009) investigated the levels of pollution indicator parameters of the water and their effects on various oxidative stress biomarkers in gill and liver tissues of spotted barb (Capoeta barroisi Lortet, 1894). The oxidative stress biomarkers analyzed included superoxide dismutase (SOD), catalase (CAT), and glucose-6-phosphate dehydrogenase (G6PD). Levels of reduced glutathione (GSH) and lipid peroxidation (LPO) were also evaluated. High levels of CAT, G6PD, GST, and GSH activity were found in the liver tissues of fish collected from the river Ceyhan discharging region. The findings of this investigation provided a rational use for oxidative stress biomarkers in aquatic ecosystem pollution biomonitoring.

In addition to their applications in the biomonitoring programs, the biochemical biomarkers are extensively used over seas as biomarker of exposure of aquatic organisms to different kind of pollutants. For example Nogueira et al. (2011) evaluated biochemical biomarkers related to oxidative stress in Nile tilapia (Oreochromis niloticus) after two and seven exposure days to diesel and pure biodiesel (B100) and blends B5 and B20 at concentrations of 0.01 and 0.1 mL L_1. Superoxide dismutase, catalase and glutathione peroxidase presented significant changes according to the treatments for all groups. They concluded that the selected stressors could activate biochemical responses in fish, at the experimental conditions tested, indicating that this fuel can also represent a risk to the aquatic biota. Also the oxidative damage and antioxidant properties of cadmium, copper, lead and zinc in chronic toxicity test as pollution biomarkers were studied in Mugel cephalus (Rajkumar et al., 2011). Increased activities of antioxidants, catalase (CAT) and glutathione-S-transferase (GST)

under long term exposures to heavy metals are more prominent to metal stress suggesting activation of physiological mechanism to scavenge the ROS produced. The results suggest that heavy metal does alter the active oxygen metabolism by modulating antioxidant enzyme activities, which can be used as biomarker to detect sub-lethal effects of pollution. Haluzova et al. (2011) investigated markers of xenobiotic metabolization (cytochrome P450,ethoxyresorufin-O-deethylase, glutathione and glutathione-S-transferase) in the liver of the common carp Cyprinus carpio after 28-day exposure to different pesticide formulations. The obtained data contributed to a better understanding of detoxification of the selected xenobitics in fish. Cohen et al. (2005) exposed Australian bass Macquaria novemaculeata to the water-accommodated fraction of Bass Strait crude oil, dispersed crude oil, or burnt crude oil to assess sub-lethal effects of oil spill remediation techniques on fish. Fish were exposed to these treatments for 16 days. Anaerobic (LDH) activity increased in the gills, liver, and white muscle after waterborne exposures. Stimulation in anaerobic activity also occurred in the liver and white muscle of fish after exposure to contaminated food. In the gills, the dispersed oil treatment resulted in the most pronounced biological response, suggesting that in the short term the use of dispersants on an oil slick might cause the most perturbations to fish metabolism.

Stress Proteins

An understanding of the effects of aquatic pollution on the biological system of the animal is important. However, information on the biochemical aspects of pollutants-binding proteins, which is necessary for a better understanding of the essential steps of the biological effects of aquatic pollution, is currently lacking (Ikebuchi et al., 1986). The exposure of living beings to sub-lethal levels of environmental pollution has been shown to trigger several defense mechanisms at the cellular levels. There is a cellular accumulation of stress proteins, which mainly act as molecular chaperones (Bauman et al., 1993; Feder & Hofmann, 1999). Cellular biomarkers such as Heat shock proteins (HSP) and Metallothionein have been extensively investigated for their use as sub-lethal indicators for environmental stress. Many aquatic organisms may, commonly experience environmental stresses such as toxic metal contamination. In order to minimize the potentially detrimental effects of these stresses, organisms are capable of synthesizing a group of proteins known as stress proteins.

In term of protein metabolism induced by heavy metals, Metallothionein are the most widely studied proteins. Metallothionein (MT) is a protein family of low molecular weight (6.0-7.0 KDa), high in the amino acid cysteine (which contains a thiol group, -SH), lack of aromatic amino acids, and ability to bend heavy metals via mercaptid linkage (Carginale et al., 1998). Although the physiological function of MT have not been fully elucidated, it has proposed that MTs have a major role in the control of intracellular metal concentration and detoxification of heavy metals and protection against reactive oxygen species; in addition, there is increasing evidence for there antioxidant function (Carginale, et al., 1998; Kagi & Schaffer, 1988). MTs are also considered to be stress proteins because they protect cells against excessive metal uptake (Bauman, et al., 1993; Kagi & Schaffer, 1988; Klaassen et al., 1999). They are found in many aquatic invertebrates and fishes. The over expression of MTs has been studied in different fish species, and their use as a biomarker for monitoring metal pollution in the environment has been proposed (Carbonell et al., 1998; Hamilton & Mehrle, 1986). The primary purpose of Metallothionein in cells is to regulate copper and zinc homeostasis and to detoxify the cell of cadmium and mercury (Klaassen, et al., 1999).

It has observed in many species that exposure to heavy metals lead to a rapid increase in MT level (Dunn et al., 1987; Schlenk et al., 1997). MT protein determination has a strong correlation with lipid peroxidation in trout chronically exposed to zinc and copper (Farag et al., 1995). Schlenk, et al. (1997) examined the effects of low-level arsenic exposure and demonstrated dose-dependent increases in MT expression in channel catfish. In aquatic invertebrates, the development of procedures for the study of MTs is relatively recent. A few studies have shown that digestive glands and gills have the highest concentrations of MTs in aquatic invertebrates (Bernal-Hernández et al., 2010; Ceratto et al., 2002; Geffard et al., 2002). Overexposure to heavy metal contaminants can lead to overproduction of MT and consequently systemic damage to the organism (Cavaletto et al., 2002; Krishnakumar et al., 1994; Lowe et al., 1995; Petrovic et al., 2001; Ringwood et al., 2004). Although many species produce Metallothionein and can be tested for metal toxicity via MT measurements, mussels demonstrated higher rates of accumulation for metals than other species because of their filter feeding and sessile life histories (Kavun et al., 2002).

Heat Shock Proteins (hsp) group belongs to a family of highly conservative proteins which are expressed in response to a wide variety of biotic and a biotic stressors and involved in protein assembly, correct folding and translocation of other cellular proteins (Hallare et al., 2004). These proteins are thought to provide the cell with protection by preventing aggregation or improper folding of proteins, thereby ensuring the survival of the organism under stressful conditions by suppressing cell damage and/or cell death. Heat shock proteins (hsps) play a pivotal role in protein homeostasis and cellular stress response within the cell (Feder & Hofmann, 1999; Iwama et al., 2004; Keller et al., 2008; Mao et al., 2005; Multhoff, 2007). Under conditions of environmental stressors, serious cellular impairments such as degradation of protein or synthesis of aberrant protein might occur. However, when cells are exposed to a stressor, the rapid activation of this gene and the subsequent synthesis of the protein have been shown to protect the cells from the harmful effect of the stressor (Morimoto et al., 1995). Organisms respond to proteotoxicity with the expression of stress proteins which are able to repair partly denatured proteins. Thus the expression of HSP is indicative of cellular changes, in particular the effects of the stressor on the protein-related machinery. Because the accumulation of these hsp has been linked to the intensity of stress, these proteins have been regarded as a suitable biomarker in assessing reactions of biota to environmental physiological stressors (Hallare, et al., 2004).

Hsp levels have been shown to be modulated in fish cells and tissues upon exposure to an array of stressors (Iwama, et al., 2004). Studies performed in low vertebrates are already numerous, and the expression of stress proteins in different fish species in response to various stressors has been investigated by many authors (Iwama et al., 1999). For instance, several hsps have been detected after the exposure of various kinds of fish cells to heat shock, arsenate and several metal ions (Currie et al., 2000; Currie et al., 1999; Misra et al., 1989). The accumulation of these hsps has been linked to the intensity of stress; these proteins have been regarded as a suitable biomarker in assessing reactions of biota to environmental and physiological stressors.

Owing to its responsiveness to diverse forms of stress, the metallothionein and heat-shock response have undergone widespread application in biomonitoring and environmental toxicology (Sanders & Dyer, 1994).

In many cases, MT and hsps are especially useful biomarkers because their induction is much more sensitive to stress than traditional indices such as growth inhibition. Kovarova & Svobodova (2009) summarized the effect of heavy metals on level of metallothionein (MT) in aquatic organisms, and evaluated that the concentrations of MT are effective indicators of Cd water pollution and explain their potential use in biomonitoring applications. Most of the literature demonstrates elevated MT and hsp levels or induction of MT and hsps under pollution conditions and then proposes MT and Hsps as a potential indicator of pollutants or toxins in the environment. For example, level of metallothionein (MT) in blue mussels was determined in order to assess the spatial distribution and temporal trends of pollution with metals in the coastal sea of Slovenia (Ramsak et al., 2012). Results revealed no significant differences between sampling sites in MT content, as well as variations in the content of Cd and Hg in mussels' tissue during the examined period. In contrast a higher expression of MT was recorded in all the fish tissues collected from wastewater-fed (contaminated) fishponds compared to fish tissues from freshwater-fed (uncontaminated) fishponds (Roy et al., 2011). They studied the potential stress that fish species are facing in wastewater-fed (contaminated) fishponds in East Calcutta Wetlands (ECWs), manifested in total protein and metallothionein (MT) concentrations. Indian major carps (IMCs) – rohu (Labeo rohita), katla (Catla catla) and mrigel (Cirrhinus mrigala) were used as suitable fish models. These findings could be important in terms of designing biomarkers for an early environmental warning system and also for monitoring fish health. Webb & Gagnon (2009) investigated Hsp70 protein in three tissue types (gill, liver, and muscle) from black bream (Acanthopagrus butcheri) collected in a highly variable estuarine environment to determine which tissue provides better inter site discrimination. The usefulness of hsp70 expression to identify anthropogenic stress under field conditions was evaluated. There was high inter fish variability in hsp70 levels in each tissue group. Ireland et al. (2004) evaluated the potential of hsp70 as a biomarker of stress produced by increased temperature, osmotic pressure, and exposure to cadmium and sodium chloride in marine macroalgae and fresh water plant species. These stressors resulted in elevated hsp70 concentrations in samples of F. serratus and L. minor when compared with unstressed controls. Results suggest that Hsp70 could potentially be applied to the detection of stress in these aquatic species, although it would probably be most effective when used in conjunction with other measurements to provide a stressor-specific biomarker profile or fingerprint. Ukamaka et al. (2010) monitored metallothionein levels over 32 days in two gastropod species Tympanotonus fuscatus and Pachymelania aurita exposed to oil coated drill cuttings. In T. fuscatus, metallothionein levels were enhanced in all treatment groups during the study. Metallothionein levels in Pachymelania aurita on the other hand was reduced in test animals exposed to the drill cuttings in comparison to their background level. The implications of the finding and possible inclusion of metallothionein in biomonitoring programmes involving the evaluation of impact of drill cuttings disposal on aquatic ecosystems are discussed. Jebali et al. (2008) quantified metallothionein in sea bass Dicentrarchus labrax intraperitoneally injected with different Cu, Cd and Hg doses after 48 h exposure. Metallothionein increased linearly with Cu and Hg doses. Cruz-Rodríguez & Chu (2002) investigated the heat shock protein response (hsp70 family) in the eastern oyster exposed to suspended clay particles spiked with polynuclear aromatic hydrocarbons (PAHs) and to suspended field contaminated sediments (SFCS). Oysters exposed to suspended clay particles spiked with PAHs showed a significant increase in Hsp70 levels, while oysters exposed to suspended unspiked clay particles did not show changes in hsp70 levels compared to the group receiving clay particles. Exposure to the SFCS resulted in a significant increase in hsp70 as a function of exposure.

These results reveal that exposure to PAHs articles and to SFCS induced a hsp70 response in the eastern oyster. Köhler et al. (2001) quantified the level of the heat shock protein (hsp70) as a biomarker of effect, in the liver of trout and loach. Laboratory experiments with different pollutant mixtures did not mimic the hsp70-inducing or inhibiting potential of field conditions, whereas effects of long-term exposure in the bypass systems showed a significant correlation with effects recorded in feral fish. Laboratory as well as semi-field studies revealed the stress response to follow an optimum curve, resulting in a maximum hsp70 level under stress but rather low hsp70 levels when stressors (chemicals, high temperature) become too severe. The study demonstrated the suitability of hsp70 stress protein levels to integrate the response dynamics of several different stressors and, therefore, to effectively function as a biomarker for the integrated effect of all environmental stressors acting on an organism (not only of chemical pollution). Rather complex kinetics of hsp70 elevation and decrease should be taken into consideration.

Aside from the biochemical biomarkers mentioned above, there are countless others. These include changes in the levels of key molecular components of the cell that can be used as an indication that an organism is under environmental stress. Such measurements include assessments of plasma vitellogenin levels, cholesterol and pyruvate concentrations (Kille et al., 1992; Lewis et al., 1999; Sanders, 1993). These compounds suffer from the fact that they respond not only to anthropogenic pollution but also physical stress and other natural phenomena. This makes them extremely difficult to be used as "stand-alone" biomarkers (Lam & Gray, 2003), and thus they will not be discussed further in this review.

Physiological Biomarkers

Physiological responses offer a major advantage for biomonitoring because the effects of pollutants usually are rapid. The effects of pollutants on physiological processes are relatively well known and include basic physiological functions as respiration, changes in growth rate, feeding, excretion osmoregulation, neurological, and heamatologicall indices (Arcand-Hoy & Benson, 1998; Bamber & Depledge, 1997; Randall et al., 1996; Wendelaar Bonga & Lock, 1992; Wood, 1992). Physiological responses are used to provide "integrated" measures of an organism's well-being, based on a range of different functional attributes. Physiological assays are especially useful for monitoring fluctuating exposures, or acting as ''early warning'' systems for acute events, because the toxic response is usually instantaneous and/or sensitive to low exposure concentrations (Handy et al., 2003).

Most physiological assays are based on recording the resting response of the organism and then quantifying changes in the physiological parameter with exposure. Growth is an important fitness component of individual organisms, and may have an overall impact on the success of natural populations. It is worth noting that although changes in a single fitness component may not always have a direct influence on the overall fitness of an individual, growth tends to integrate and reflect most sub-lethal effects. Growth has, therefore, been widely used as an indicator of pollution stress in marine invertebrates, e.g. bivalves (Page & Widdows, 1991; Widdows et al., 1982) and gastropods (Wo et al., 1999) providing a measure of environmental quality. Scope for growth has been employed in a number of pollution monitoring investigations. For example, samples of the giant mussel, Choromytilus chorus, were collected at three sampling stations exposed to different degrees of pollution along the south–central portion of the Chilean coast in 1998 and 1999 (Toro et al., 2003).

Scope for growth (SFG) was employed as a physiological index to evaluate stress produced by pollutants existing at each sampling site. Individuals from San Vicente bay (highly polluted) showed negative SFG values, possibly indicating severe stress related to the accumulation of toxic compounds in their tissues. Indeed, there was a significant negative correlation between the SFG of the different populations of C. chorus and the concentrations of organochlorines (OCs) and polycyclic aromatic hydrocarbons (PAHs) in their tissues (Toro, et al., 2003). Respiratory responses have been used to monitor pollutant stress in invertebrates and fishes. These are rapid and therefore useful for identifying short pollution events. Oxygen consumption rate is mainly used because it shows a clear dose-response in many organisms and for many chemicals and is also a surrogate for metabolic rate (McKim & Erickson, 1991; Randall, et al., 1996). Handy, et al. (2003) reviewed the utility of physiological measurements as biomarkers of pollutant exposure and biological effect, and then gives a step by step description of methodologies used to measure physiological parameters in mostly fish and invertebrates. The effects of pollutants on respiratory, cardiovascular, osmoregulatory, and neuro-endocrine processes are relatively well described in laboratory experiments and some of these responses particularly ventilation, heart rate, and also body ion fluxes have been evaluated as biomonitors or potential biomarkers in the field. They concluded that physiological assays are especially useful for monitoring fluctuating or complex exposures, or acting as ‘‘early warning’’ systems for acute events.

Since blood parameters are influenced by a variety of environmental stressors, they have the potential to be used as biomarkers. Hematological techniques are the most common method to determine the sub-lethal effects of the pollutants (Larsson et al., 1995). In fish, exposure to chemical pollutants can induce either increases or decreases in hematological levels (Kori-Siakpere et al., 2006). Hematological parameters are closely related to the response of the animal to the environment, an indication that the environment where fishes live could exert some influence on the hematological characteristics (Gabriel et al., 2004). Blood is a good indicator to determine the health of an organism and hematological parameters are important in diagnosing the functional status of exposed animals to toxicants (Joshi et al., 2002). As a result of association between the circulatory system and the external environment, the hematological variables were used to determine the effects of external stressors and toxic substances (Wendelaar Bonga & Lock, 1992). It has been suggested that hematology of fish be used in pollutants toxicity detection (Wepener et al., 2005). Damage to blood and hemopoietic organs in fish may be associated due to either change in environmental conditions (Gardner & Yevich, 1969) or water born pollutants (Dawson, 1935; Gardner & Yevich, 1969; Reichenbach-Klink, 1966). Blood analysis is crucial in the area of toxicology and environmental monitoring as possible indicator of physiological changes in fishery management and diseases investigation. The most common hematological variables measured during stress included RBC (Red Blood Cells), HGB (Hemoglobin), HCT (Hematocrit), MCV (Mean Cellular Volume), MCH (Mean Cellular Hemoglobin Concentration), PLT (Thrombocytes) (Wedemeyer & Yasutake, 1977). Many studies have demonstrated changes in blood variables as a result of environmental conditions such as temperature (Houston & Pilar Schrapp, 1994), radiation (Schultz et al., 1993), hypoxia (Scott & Rogers, 1981), and presence of contaminants (Houston & Pilar Schrapp, 1994). As an indicator of pollution, blood parameters are used in order to diagnose and describe the general health condition of some fish (Kori-Siakpere, et al., 2006; Maheswaran et al., 2008; Rogers et al., 2003). Besides, this type of index reflects certain ecological changes in the environment (Roche & Boge, 1996).

Blood biochemical variables such as liver function tests are usually recognized as the reliable indicator of liver metabolism (Tseng et al., 1988). The raised enzymatic activity in the liver may be because of induction of enzyme synthesis (Kimbrough et al., 1971; Krample & Hladka, 1975; Street, 1969), while their low levels could either be due to enzymatic inhibition (Henderson, 2005; Meany & Pocker, 1979) or due to liver damage without any regeneration. The main hepatic cellular component to be affected by the ambient toxicants seems to be the cell membrane. Aquatic toxicants either have increased the membrane permeability causing enhanced leaching out of the enzymes, or reduced the permeability forcing the enzymes to accumulate in the cells. Cellular damage is another reason for decreased synthesis of enzymes in living organisms. Monitoring of liver enzymes leakage into the blood has proved to be a very useful tool in liver toxicological studies.

Little information is available about the potential application of physiological biomarkers under field conditions, particularly in free living fish populations. The use of physiological biomarkers to identify pollutant exposure in the field is relatively new to ecotoxicology. For example Yousafzai & Shakoori (2011) evaluated freshwater fish, Tor putitora caught from polluted portion of river Kabul for various physiological parameters and was compared with control fish caught from non polluted Warsak Dam to know the possible toxic effects of pollution in the river. The selected variable exhibited remarkable alterations in the fishes collected from polluted site. The increase and decrease in various physiological and biochemical parameters in the liver of test fish samples in comparison with the control shows the adverse effect of aquatic pollution on the fish health. McKenzie et al. (2007) used portable swimming respirometers to compare respiratory metabolism of fish exposed in cages for three weeks to either clean or polluted sites on three urban European river systems: the river Lambro, Milan, Italy; the rivers Blythe, Cole and Tame, Birmingham, UK; and the river Amstel, Amsterdam, The Netherlands. Measurements of oxygen uptake during swimming revealed increased rates of routine aerobic metabolism in both chub and carp at polluted sites in all of the rivers studied, indicating a sub-lethal metabolic loading effect. Therefore, the physiological traits of exercise performance and metabolic rate have potential as biomarkers of the overall sub-lethal toxic effects of exposure to complex mixtures of pollutants in rivers, and may also provide insight into why fish do not colonize some polluted environments. As a part of monitoring program, Linderoth et al. (2006) measured a battery of basic physiological biomarkers in adult female perch (Perca fluviatilis), an assumed aquatic pollution gradient was confirmed, with the city of Stockholm (Sweden) as a point source of anthropogenic substances. The results indicated a severe pollution situation in central Stockholm, with poor health status of the perch. Besides the main gradient other sources of pollution also influenced the response pattern of the measured biomarkers. In particular, there were strong indications of pollution coming from the Baltic Sea. In the same way, Camargo & Martinez (2006) evaluated from a set of biochemical and physiological biomarkers the ones which could work as sensitivity tools for the environmental quality assessment. In situ tests were carried out at three sites along an urban stream heavily contaminated by anthropogenic activities and at a reference site. The variables analyzed were: hemoglobin content (Hb), plasma concentrations of cortisol, glucose, total protein, Na+ and Cl−, plasma osmolarity, liver activities of glutathione-S-transferase (GST) and catalase and interrenal cells area. Results showed that glycemia, interrenal cell size and GST activity, which were significantly higher in fish caged in the urban stream, were best able to distinguish between the most disturbed sites and the reference one, showed to be a promising tool for the assessment and monitoring of tropical aquatic ecosystems.

Some new variables as plasmatic parameters in fish were evaluated to investigate whether they could serve as physiological indicators to evaluate water quality (Masson et al., 2002). Plasma Cl content in two fish species caught in a wide range of rivers representative of the hydrographic system of Lorraine (N-E France) were investigated. While indigenous trout maintained their Cl content even in the acidic streams, transferred trout exhibited an important decrease of Cl content after 48 h of exposure under acidic conditions.

Some blood parameters have also proved to be quiet sensitive toward many toxicants in laboratory experiments (Lauren & McDonald, 1985; Patrick & Wood, 1999; Poleeo et al., 1995). For example, Adedeji et al. (2009) assessed the effect of diazinon on cultured and wild African catfish (Clarias gariepinus). Examination of erythrocytes and leukocytes profile was performed after 96 h of exposure to DiazintolR. The experimental group of the African catfish showed significantly lower values of erythrocytes count (RBC), hemoglobin content (Hb) and hematocrit (PCV) compared to the control group. On the contrary, there was a significant decrease in leukocyte count ($p < 0.05$), as well as in both the relative and absolute lymphocyte count. Changes in values of both the erythrocytes and leukocytes profile after exposure to diazinon-based preparation may be referred to disruption of hematopoiesis as well as to a decrease on non-specific immunity of the fish. As another example of exposure Parma et al. (2007) exposed freshwater fish Prochilodus lineatus to sublethal concentrations of cypermethrin for 2, 5 and 8 days. It was observed that with the increase of exposure time total erythrocyte (RBC), hemoglobin (Hb), hematocrit (Ht) and mean corpuscular hemoglobin concentration (MCHC) values decreased. These reports indicate that hematological parameters, may be useful as a diagnostic test for cypermethrin exposure in aquatic organisms.

The heavy metals are the commonly used toxicant in the exposure experiments. For example Serezli et al. (2011) evaluated whether short-term exposures (3 h) to high concentrations of heavy metals may induce blood cells in Coruh trout (Salmo coruhensis). It was investigated that copper and lead have effects on hematocrit, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic and pyruvic transaminase (SGPT), total protein, blood cell numbers and erythrocyte morphology of S. coruhensis exposed to two copper and lead concentrations for 3 h. In the same way Vinodhini & Narayanan (2009) investigated the effect of heavy metal pollutants such as cadmium, chromium, nickel and lead in aquatic system on common carp (Cyprinus carpio L.) by using a set of biochemical parameters. Concentrations of red blood cells, blood glucose and total cholesterol were significantly elevated. The study suggested that the presence of toxic heavy metals in aquatic environment has strong influence on the hematological parameters in the fresh water fish common carp (Cyprinus carpio L.). Also, Adeyemo (2007) investigated the changes in Clarias gariepinus' blood cells after 96-h of exposure to lead. The packed cell volume (PCV) of the treatments decreased significantly relative to that of the control, while their platelet counts increased compared to the control. There was also a reduction in the RBC of treatments. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) increased considerably in all treatments compared to the control. These alterations have been attributed to direct or feedback responses of structural damage to RBC membranes resulting in haemolysis and impairment in hemoglobin synthesis, stress related release of RBCs from the spleen and hypoxia, which was induced by exposure to lead (Adeyemo, 2007).

References

1. Connell, D., Lam, P., Richardson, B. & Wu, R. (1999). Introduction to Ecotoxicology. UK., Blackwell Science Ltd,.

2. Lam, P. K. S. & Gray, J. S. (2003). The use of biomarkers in environmental monitoring programmes. . Marine Pollution Bulleten 46: 182–186.
3. Jimenez, B. D. & Stegeman, J. J. (1990). Detoxication enzymes as indicators of environmen.ta1 stress in fish. In: Adams, S. M. (ed.) Biological indicators of stress in fish. American Fisheries Society Symposium 8: 67-79.
4. Barnhoorn, I. E. J. & Van Vuren, J. H. J. (2004). The use of different enzymes in feral freshwater fish as a tool for the assessment of water pollution in South Africa. Ecotoxicology and Environmental Safety 59: 180-185.
5. Powers, D. A. (1989). Fish as model systems. Science (New York, N.Y.) 246(4928): 352-358.
6. Gayet, J. C., Haouz, A., Gelosomeyer, A. & Burstein, C. (1993). Detection of heavy-metal salts with biosensors built with an oxygen-electrode coupled to various immobilized oxidases and dehydrogenases. Biosensors and Bioelectronics 8: 177-183.
7. Zollner, h. (1993). Handbook of enzyme inhibitors, VCH,Weinheim, Basel, New York.
8. Melancon, M. J. (1995). Bioindicators used in aquatic and terrestrial monitoring, Lewis Publishers, Boca Raton, FL.
9. Casillas, E., Myers, M. & Ames, W. E. (1983). Relationship of serum chemistry values to liver and kidney histopathology in english sole (Parophrys vetulus) after acute exposure to carbon-Tetrachloride. Aquatic Toxicology 3(1): 61-78.
10. Heath, A. (1996). Water pollution and fish physiology, Boca Raton, Lewis Publs.
11. Moon, T. W. & Mommsen, T. P. (1987). Enzymes of intermediary metabolism in tissues of the little skate. Journal of Experimental Zoology 244: 9-15.
12. Kiessling, A., Kiessling, K. H., Storebakken, T. & Asgard, T. (1991). Changes in the structure and function of the epaxial muscle of rainbow trout (Oncorhynchus mykiss) in relation to ration and age .2. Activity of key enzymes in energy metabolism. Aquaculture 93(4): 357-372.
13. Cowey, C. B., Cooke, D. J., Matty, A. J. & Adron, J. W. (1981). Effects of quantity and quality of dietary protein on certain enzyme activities in rainbow trout. The Journal of nutrition 111(2): 336-345.
14. Hilton, J. W. & Atkinson, J. L. (1982). Response of rainbow trout (Salmo gairdneri) to increased levels of available carbohydrate in practical trout diets. The British journal of nutrition 47(3): 597-607.
15. Pelletier, D., Dutil, J. D., Blier, P., Guderley, H. & Physiology, B. (1994). Relation between growth rate and metabolic organization of white muscle, liver and digestive-tract in Cod, Gadus morhua. Journal of Comparative Biochemical Systemic and Environmental Physiology 164: 508-508.
16. Segner, H. & Verreth, J. (1995). Metabolic enzyme activities in larvae of the African catfish, Clarias gariepinus - changes in relation to age and nutrition. Fish physiology and biochemistry 14(5): 385-398.
17. Livingstone, D. R., Lemaire, P., Matthews, A., Peters, L. D., Porte, C., Fitzpatrick, P. J., Forlin, L., Nasci, C., Fossato, V., Wootton, N. & Goldfarb, P. (1995). Assessment of the impact of organic pollutants on Goby (Zosterisessor ophiocephalus) and Mussel (Mytilus galloprovincialis) from the Venice Lagoon, Italy: biochemical studies. Marine Environmental Research 39: 235-240.
18. Fitzpatrick, P. J., O'Halloran, J., Sheehan, D. & A.R., W. (1997). Assessment of a glutathione S-transferase and related proteins in the gill and digestive gland of Mytilus edulis (L.), as potential organic pollution biomarkers. Biomarkers 2: 51-56.

19. Osman, A. G., Abd El Reheem, A. M., AbuelFadl, K. Y. & GadEl-Rab, A. G. (2010). Enzymatic and histopathologic biomarkers as indicators of aquatic pollution in fishes. Natural Science 2(11): 1302-1311.
20. Osman, A. G. (2012). Biomarkers in Nile tilapia Oreochromis niloticus niloticus (Linnaeus, 1758) to assess the impacts of river Nile pollution: bioaccumulation, biochemical and tissues biomarkers. Journal of Environmental Protection 3: 966-977.
21. Sahan, A., Belge, E. & Altun, T. (2010). The Determination of Biochemical Indicators (Biomarkers) in the Common Carp (Cyprinus carpio) to the Physico-chemical Parameters of the Ceyhan River (Adana-Turkey). Ekoloji 19: 8-14.
22. Tsangaris, C., Cotou, E., Papathanassiou, E. & Nicolaidou, A. (2010). Assessment of contaminant mpacts in a semi-enclosed estuary (Amvrakikos Gulf, NW Greece): Bioenergetics and biochemical biomarkers in mussels. Environmental Monitoring and Assessment 161: 259-269.
23. Jemec, A., Drobne, D., Tišler, T. & Sepčić, K. (2009). Biochemical biomarkers in environmental studies—lessons learnt from enzymes catalase, glutathione Stransferase and cholinesterase in two crustacean species. Environmental Science and Pollution Research 17: 571-581.
24. Kurutas, B. E., Sahan, A. & Altun, T. (2009). Oxidative stres biomarkers in liver and gill tissues of spotted barb (Capoeta barroisi L, 1894) living in the River Ceyhan, Adana, Turkey. Turkish Journal Biolology 33: 275-282.
25. Nogueira, L., Rodrigues, A., Trídico, C., Fossa, C. & de Almeida, E. (2011). Oxidative stress in Nile tilapia (Oreochromis niloticus) and armored catfish (Pterygoplichthys anisitsi) exposed to diesel oil. Environmental Monitoring and Assessment 180: 243-255.
26. Rajkumar, J. S. I., John Milton, M. C., Ulthiralingam, M., Azhaguraj, R., Ganesh, J. & Ambrose, T. (2011). Toxic effects and bioaccumulation of cadmium, copper, lead and zinc in post larval stages of Penaeus monodon. International Journal of Development Research 1: 1-5.
27. Haluzova, I., Modra, H., Blahova, J., Havelkova, M., Široka, Z. & Svobodova, Z. (2011). Biochemical markers of contamination in fish toxicity tests. Interdisciplinary Toxicology 4: 85–89.
28. Cohen, A., Gagnon, M. & Nugegoda, D. (2005). Alterations of Metabolic Enzymes in Australian Bass, Macquaria novemaculeata, After Exposure to Petroleum Hydrocarbons. Archives of Environmental Contamination and Toxicology 49: 200–205.
29. Ikebuchi, H., Teshima, R., Suzuki, K., Terao, T. & Yamane, Y. (1986). Simultaneous induction of Pb- metallothione like protein and Zn- thioneine in the liver of rats given lead acetate. Biochemestry Journal 233: 541-546.
30. Bauman, J. W., Liu, J. & Klaassen, C. (1993). Production of metallothioneins and heat shock proteins in response to metals. Fundamental Applied Toxicology 21: 15-22.
31. Feder, M. E. & Hofmann, G. E. (1999). Heat shock proteins, molecular chaperones and stress response: Evolutionary and ecological physiology. Annual Review of Physiology 61: 243-282.
32. Carginale, V., Scudiero, R., Capasso, C., Capasso, A., Kille, P., di Prisco, G., Parisi, E. & Biochem, J. (1998). Cadmium-induced differential accumulation of metallothionein isoforms in the Antarctic icefish, which exhibits no basal metallothionein protein but high endogenous mRNA levels. Biochemical Journal 332: 475–481.
33. Kagi, J. H. & Schaffer, A. (1988). Biochemistry of metallothionein. Biochemistry 27: 8509-8515.
34 Klaassen, C. D., Liu, J. & Choudhuri, S. (1999). Metallothionein: An intracellular protein to protect against cadmium toxicity. Annual Review of Pharmacology and Toxicology 39: 267-274.

35 Hamilton, S. J. & Mehrle, P. M. (1986). Metallothionein in fish: Review of its importance in assessing stress from metal contaminants. Transactions of the American Fisheries Society 115: 596-609.

36 Carbonell, G., Martinez-Pereda, J. & Tarazona, J. (1998). Mobilization of essential metals during and after short-term lethal cadmium exposure in rainbow trout (Oncorhynchus mykiss). Ecotoxicology and Environmental Restoration 1: 85-91.

37 Dunn, M. A., Blauck, T. L. & Cousins, R. J. (1987). Metallothionein. Proceedings of the Society for Experimental Biology and Medicine 185: 107-119.

38 Schlenk, D., Chelius, M., Wolford, L., Khan, S. & Chan, K. M. (1997). Characterization of hepatic metallothionein expression in channel catfish (Ictalurus punctatus) by reverse transcriptase polymerase chain reaction. Biomarkers 2: 161–167.

39 Farag, A. M., Stansbury, M. A., Hogstrand, C., MacConnell, E. & Bergman, H. L. (1995). The physiological impairment of free ranging brown trout exposed to metals in the Clark Fork River, Montana. Canadian Journal of Fisheries and Aquatic Sciences 52: 2038-2050.

40 Ceratto, N., Dondero, F., Van de Loo, J. W., Burlando, B. & Viarengo, A. (2002). Cloning and sequencing of a novel metallothionein gene in Mytilus galloprovincialis Lam. Comparative Biochemistry and Physiology C 131: 217–222.

41 Geffard, A., Amiard, J. C. & Amiard-Triquet, C. (2002). Use of metallothionein in gills from oysters (Crassostrea gigas) as a biomarker: seasonal and intersite fluctuations. Biomarkers 7: 123–137.

42 Bernal-Hernández, Y. Y., Medina-Díaz, I. M., Robledo-Marenco, M. L., Velázquez-Fernández, J. B., Girón-Pérez, M. I., Ortega-Cervantes, L., Maldonado-Vázquez, W. A. & Rojas-García, A. E. (2010). Acetylcholinesterase and metallothionein in oysters (Crassostrea corteziensis) from a subtropical Mexican Pacific estuary. Ecotoxicology 19(4): 819-825.

43 Krishnakumar, P. K., Casillas, E. & Varanasi, U. (1994). Effect of Environmental Contaminants on the Health of Mytilus edulis from Puget-Sound, Washington, USA .1. Cytochemical Measures of Lysosomal Responses in the Digestive Cells Using Automatic Image-Analysis. Marine Ecology-Progress Series 106: 249-261.

44 Lowe, D. M., Fossato, U. V. & Depledge, M. H.-. (1995). Contaminant-induced lysosomal membrane damage in blood cells of mussels Mytilus galloprovincialis from the Venice Lagoon: an in vitro study. Marine Ecology Progress Series 129: 189-196.

45 Petrovic, S., Ozretic, B., Krajnovic-Ozretic, M. & Bobinac, D. (2001). Lysosomal membrane stability and metallothioneins in digestive gland of mussels (Mytilus galloprovincialis Lam.) as biomarkers in a field study. Marine Pollution Bulletin 42: 1373-1378.

46 Cavaletto, M., Ghezzi, A., Burlando, B., Evangelisti, V., Ceratto, N. & Viarengo, A. (2002). Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of Mytilus galloprovincialis. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 131: 447-455.

47 Ringwood, A. H., Hoguet, J., Keppler, C. & Gielazyn, M. (2004). Linkages between cellular biomarker responses and reproductive success in oysters - Crassostrea virginica. Marine Environmental Research 58: 151-155.

48 Kavun, V. Y., Shulkin, V. M. & Khristoforova, N. K. (2002). Metal accumulation in mussels of the Kuril Islands, north-west Pacific Ocean. Marine Environmental Research 53: 219-226.

49 Hallare, A. V., Kohler, H. R. & Triebskorn, R. (2004). Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56(7): 659-666.

50 Iwama, G. K., Afonso, L. O. B., Todgham, A., Ackerman, P. & Nakano, K. (2004). Are hsps suitable for indicating stressed states in fish? Journal of Experimental Biology Commentary 207(15-19).

51 Mao, L., Bryantsev, A. L., Chechenova, M. B. & Shelden, E. A. (2005). Cloning, characterization, and heat stressinduced redistribution of a protein homologous to human Hsp27 in the zebrafish Danio rerio. Experimental Cell Research 306: 230-241.

52 Multhoff, G. (2007). Heat shock protein 70 (Hsp70): Membrane location, export and immunological relevance. Methods 43: 229-237.

53 Keller, J. M., Escara-Wilke, F. & Keller, E. T. (2008). Heat stress-induced heat shock protein 70 expression is dependent on ERK activation in zebrafish (Danio rerio) cells. Comparative Biochemistry and Physiology A 150: 307-314.

54 Morimoto, R., Cotto, J., Kline, M., Kroeger, P., Lee, B., Satyal, S. & Shi, Y. (1995). The Heat-Shock Response - Sensing and Responding to Changes in the Environment. Journal of Cellular Biochemistry: 188.

55 Iwama, G. K., Vijayan, M. M., Forsyth, R. B. & Ackerman, B. A. (1999). Heat shock proteins and physiological stress in fish. American Zoologist 39: 901-909.

56 Misra, S., Zafarullah, M., Price-Haughey, J. & Gedamu, L. (1989). Analysis of stress induced gene expression in fish cell lines exposed to heavy metals and heat shock. Biochimica et Biophysica Acta 1007: 325-333.

57 Currie, S., Tufts, B. L. & Moyes, C. D. (1999). Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. American Journal of Physiology 276: R990-R996.

58 Currie, S., Moyes, C. D. & Tufts, B. L. (2000). he eVects of heat shock and acclimation temperature on Hsp70 and Hsp30 mRNA expression in rainbow trout: In vivo and in vitro comparisons. Journal of Fish Biology 56: 398-408.

59 Sanders, B. M. & Dyer, S. D. (1994). Cellular stress response. Environmental Toxicology and Chemistry 13: 1209-1210.

60 Kovarova, J. & Svobodova, Z. (2009). Can thiol compounds be used as biomarkers of aquatic ecosystem contamination by cadmium? Interdisciplinary Toxicology 2: 177–183.

61 Ramsak, A., Stopar, K. & Malej, A. (2012). Comparative phylogeography of meroplanktonic species, Aurelia spp. and Rhizostoma pulmo (Cnidaria: Scyphozoa) in European Seas. hydrobiologia 690: 69-80.

62 Roy, U. S., Chattopadhyay, B., Datta, S. & Mukhopadhyay, S. K. (2011). Metallothionein as a Biomarker to Assess the Effects of Pollution on Indian Major Carp Species from Wastewater-Fed Fishponds of East Calcutta Wetlands (a Ramsar Site). Environmental Research, Engineering and Management 4: 10-17.

63 Webb, D. & Gagnon, M. M. (2009). The value of stress protein70 as an environmental biomarker of fish health under field conditions. Environmental Toxicology 24: 287–295.

64 Ireland, H. E., Harding, S. J., Bonwick, G. A., Jones, M., Smith, C. J. & Williams, J. H. H. (2004). Evaluation of heat shock protein 70 as a biomarker of environmental stress in Fucus serratus and Lemna minor. Biomarkers 9: 139-155.

65 Ukamaka, A. M., Obinnaya, C. L., Adebayo, O. & Miriam, L. (2010). Metallothionein induction in edible mangrove periwinkles, Tympanotonus fuscatus var radula and Pachymelania aurita exposed to Oily Drill Cuttings. Journal of American Science 6: 89-97.

66 Jebali, J., Banni, M., Gerbej, H., Boussetta, H., López-Barea, J. & Alhama, J. (2008). Metallothionein induction bu Cu, Cd and Hg in Dicentrarchus labrax liver: assessment by RP-HPLC with fluorescent detection and spectrophotometry. Marine Environmental Research 65: 358-363.

67 Cruz-Rodríguez, L. A. & Chu, F. L. (2002). Heat-shock protein (HSP70) Response in the Eastern Oyster, Crassostrea virginica, Exposed to PAHs Sorbed to Suspended Artificial Clay Particles and to Suspended Field Contaminated Sediments. Aquatic Toxicology 60: 157-168.

68 Köhler, H.-R., Bartussek, C., Eckwert, H., Farian, K., Gränzer, S., Knigge, T. & Kunz, N. (2001). The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. Journal of Aquatic Ecosystem Stress and Recovery 8: 261-279.

69 Kille, P., Kay, J. & Leaver, M. (1992). Induction of piscine metallothionein as a primary response to heavy metal pollutants: applicability of new sensitive molecular probes. Aquatic Toxicology 22: 279-286.

70 Sanders, B. M. (1993). Stress proteins in aquatic organisms: An environmental perspective. Critical Reviews in Toxicology 23: 49-75.

71 Lewis, S., Handy, R. D., Cordi, B., Billinghurst, Z. & Depledge, M. H. (1999). Stress proteins (HSP's): Methods of Detection and Their Use as an Environmental Biomarker. Ecotoxicology 8: 351-368.

72 Wendelaar Bonga, S. E. & Lock, R. A. C. (1992). Toxicans and osmoregulation in fish. Netherlands Journal of Zoology 42: 478-493.

73 Wood, C. M. (1992). Flux measurements as indices of Hq and metal effects on freshwater fish. Aquatic Toxicology 22: 239-246.

74 Randall, D. J., Brauner, C. J., Thurston, R. V. & Neuman, J. F. (1996). Water chemistry at the gill surfaces of fish and the uptake of xenobiotics. Toxicology of Aquatic Pollution. Taylor, E. W., Cambridge: University Press: 1-16.

75 Bamber, S. D. & Depledge, M. H. (1997). Responses of shore crabs to physiological challenges following exposure to selected environmental contaminants. Aquatic Toxicology 40: 79-92.

76 Arcand-Hoy, L. D. & Benson, W. H. (1998). Fish reproduction: An ecologically relevant indicator of endocrine disruption. Environmental Toxicology and Chemistry 17: 49-57.

77 Handy, R. D., Galloway, T. S. & Depledge, M. H. (2003). A Proposal for the Use of Biomarkers for the Assessment of Chronic Pollution and in Regulatory Toxicology. Biomarkers 12: 331-343.

78 Widdows, J., Bakke, T., Bayne, B. L., Donkin, P., Livingstone, D. R., Lowe, D. M., Moore, M. N., Evans, S. L. & Moore, S. L. (1982). Responses of Mytilus edulis on exposure to the water-accommodated fraction of North Sea oil. Marine Biology 67: 15-31.

79 Page, D. S. & Widdows, J. (1991). Temporal and spatial variation in levels of alkyltins in mussel tissues: A toxicological interpretation of field data. Marine Environmental Research 32: 113-129.

80 Wo, K. T., Lam, P. K. S. & Wu, R. S. S. (1999). A comparison of growth biomarkers for assessing sublethal effects of cadmium on a marine gastropod, Nassarius festivus. Marine Pollution Bulleten 39: 165-173.

81 Toro, B., Navarro, J. M. & Palma-Fleming, H. (2003). Relationship between bioenergetics responses and organic pollutants in the giant mussel, Choromytilus chorus (Mollusca: Mytilidae). Aquatic Toxicology 63: 257-269.

82 McKim, J. M. & Erickson, R. J. (1991). Environmental impacts on the physiological mechanisms controlling xenobiotic transfer across fish gills. Physiological Zoology 64: 39-67.

83 Larsson, A., Haux, C. & Sjobeck, M. (1995). Fish physiology and metal pollution: Results and experiences from laboratory and field studies. Ecotoxicology and environmental safety 9: 250-281.
84 Kori-Siakpere, O., Ake, J. E. G. & Avworo, U. M. (2006). Sub-lethal effects of some selected haematological parameters of Heteroclarias (A hybrid of Heterobranchus bidorsalis and Clarias gariepinus). International Journal of Zoological Research 2: 77-83.
85 Gabriel, U. U., Ezeri, G. N. O. & Opabunmi, O. O. (2004). Influence of sex, source, health status and acclimation on the haematology of Clarias gariepinus (Burch, 1822). African Journal of Biotechnology 3: 463-467.
86 Joshi, P. K., Bose, M. & Harish, D. (2002). Changes in certain haematological parameters in a siluroid catfish Clarias batrachus (Linn) exposed to cadmium chloride. Pollution Resources 21: 129 - 131.
87 Wepener, V., Van Vuren, J., Chartiza, F., Slabbert, L. & Masolab, B. (2005). Active biomonitoring in freshwater environments: early warning signals from biomarkers in assessing biological effects of diffuse sources of pollutants. Physics and Chemistry of the Earth 30: 751-761. .
88 Gardner, G. R. & Yevich, P. P. (1969). Studies on the blood morphology of three estuarine Cyprinodontiform fishes. Journal of the Fisheries Research Board of Canada 26: 433–437.
89 Dawson, A. B. (1935). The hemopoietic response in the catfish Ameittrus nebulosus to chronic lead poisoning. The Biological Bulletin 68: 335–346.
90 Reichenbach-Klink, H. H. (1966). The blood components of fish with relation to parasiyies infection and water pollution. Bull Off Intern Epizoo 65: 1039–1054.
91 Wedemeyer, C. A. & Yasutake, W. T. (1977). Clinical methods for the assessment of the effects of environmental stress on fish health. United States Technical Papers and United States Fish Wildlife Services 89: 1-18.
92 Houston, H. & Pilar Schrapp, M. (1994). Thermoacclimatory hematological response: Have we been using appropriate conditions and assessment methods? . Canadian Journal of Zoology 72: 1238-1242.
93 Schultz, N., Norrgren, L., Grawe, J., Johannisson, A. & Medhage, O. (1993). Micronuclei frequency in circulating erythrocytes from rainbow trout (Oncorhynchus mykiss) subjected to radiation, an image analysis and flow cytometric study. Comparative Biochemistry and Physiology C 105: 207-211.
94 Scott, A. L. & Rogers, W. A. (1981). Haematological effects of prolonged sublethal hypoxia on channel catfishIctalurus punctatus (Rafinesque. Journal of Fish Biology 18: 591–601.
95 Rogers, J. T., Richards, J. G. & Wood, C. M. (2003). Ion regulatory disruption as the acute toxic mechanism for lead in the rainbow trout. Aquatic Toxicology 64: 215-234.
96 Maheswaran, R., Devapanl, A., Muralidharan, S., Velmurugan, B. & Ignaeimuthu, S. (2008). Haematological studies of fresh water fish, Clarias batradrus (L) exposed to mercuric chloride. IJIB 2: 49-54.
97 Roche, H. & Boge, G. (1996). Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. Marine Environmental Research 41: 27-43.
98 Tseng, C., Leng, F. W., Chang, E. E. & Yih-shih, T. U. N. (1988). Studies on chronic cadmium intoxication. Journal of Clinical Biochemistry 17: 32-41.
99 Street, J. C. (1969). Organochlorinated insecticides and the stimulation of liver microsome enzyme. Annals of the New York Academy of Sciences 160: 274-290.

100 Kimbrough, R. D., Gaines, T. B. & Linder, R. E. (1971). The ultrastructure of livers of rats fed DDT and dieldrin. Archives of Environmental Health 22: 460-467.
101 Krample, V. & Hladka, A. (1975). Dose dependent extent of microsomal enzyme induction by aldrin and dieldrin in rats. Bulletin of Environmental Contamination and Toxicology 14: 571-557.
102 Meany, J. E. & Pocker, Y. (1979). The in vivo inactivation of lactate dehydrogenase by organochlorine insecticides. Pesticide Biochemistry and Physiology 11: 232-242.
103 Henderson, R. E. (2005). Biomarker Human Health. Enciclopedia of Toxicology. P, W. New York, Elselvier.
104 Yousafzai, A. M. & Shakoori, A. R. (2011). Hepatic Responses of A Freshwater Fish Against Aquatic Pollution. Pakistan Journal of Zoology 43: 209-221.
105 McKenzie, D. J., Garofalo, E., Winter, M., Ceradini, S., Verweij, F., Hayes, R., van der Oost, R., Butler, P., Chipman, J. & Taylor, E. W. (2007). Complex physiological traits as biomarkers of the sub-lethal toxicological effects of pollutant exposure in fishes. Philosophical Transaction of Royal Society London B Biological Science 362: 2043–2059.
106 Linderoth, M., Hansson, T., Liewenborg, B., Sundberg, H., Noaksson, E., Hanson, M., Zebühr, Y. & Balk, L. (2006). Basic Physiological Biomarkers in Adult Female Perch (Perca Fluviatilis) in a Chronically Polluted Gradient in the Stockholm Recipient (Sweden). Marine Pollution Bulletin 53: 437-450.
107 Camargo, M. M. & Martinez, C. B. R. (2006). Biochemical and physiological biomarkers in Prochilodus lineatus submitted to in situ tests in an urban stream in southern Brazil. Environmental Toxicology and Pharmacology 21: 61-69.
108 Masson, N., Guerold, F. & Dangles, O. (2002). Use of blood parameters in fish to assess acidic stress and chloride pollution in French running waters. Chemosphere 47: 467-473.
109 Lauren, D. J. & McDonald, D. G. (1985). Effects of copper on branchial ionoregulation in the rainbow trout, Salmo gairdneri Richardson. Journal of comparative Physiology 155: 636-644.
110 Poleeo, A. B. S., Oxnevad, S. A., Ostbye, K., Andersen, R. A., Oughton, D. H. & Vollestad, L. A. (1995). Survival of crucian carp (Carassius carassius) exposed to a high low-molecular weight inorganic aluminium challenge. Aquacultural Science 57: 350–359.
111 Patrick, M. L. & Wood, C. M. (1999). Ion and acid-base regulation in the freshwater mummichog (Fundulus heteroclitus): a departure from the standard model for freshwater teleost. Comparative Biochemistry and Physiology 122: 445–456.
112 Adedeji, O. B., Adedeji, O. A., Adeyemo, O. K. & Agbede, S. A. (2009). Acute Effects Of Diazinon On Blood Paramters In The African Catfish (Clarias Gariepinus). The Internet Journal of Hematology 5: 2.
113 Parma, M. J., Loteste, A., Campana, M. & Bacchetta, C. (2007). Changes of hematological parameters in Prochilodus lineatus (Pisces, Prochilodontidae) exposed to sublethal concentration of cypermethrin. Journal of Environmental Biology 28: 147-149.
114 Serezli, R., Akhan, S. & Delihasan-Sonay, F. (2011). Acute effects of copper and lead on some blood parameters on Coruh trout (Salmo coruhensis). African Journal of Biotechnology 10: 3204-3209.
115 Vinodhini, R. & Narayanan, M. (2009). The impact of toxic heavy metals on the hematological parameters in common carp (Cyprinuscaprio L.) Iranian Journal of Environmental Health Science & Engineering 6: p23.
116 Adeyemo, O. K. (2007). Haematological profile of Clarias gariepinus (Burchell,1822) exposed to lead. Turkish Journal of Fisheries and Aquatic Sciences 7: 163-169.