INTRODUCTION

Biochemical and physiological biomarkers have been used in order to prevent irreversible damage in whole organisms, communities and ecosystems (López-Barea and Pueyo, 1998). Measurement of biochemical and physiological parameters is a commonly used diagnostic tool in aquatic toxicology and biomonitoring. The impact of a number of contaminants on aquatic ecosystems can be assessed by the measurement of their external levels in the surrounding water or sediments, or by determining some biochemical parameters in fish and other organisms that respond specially to the degree and type of contamination (Petrivalsky et al., 1997; Machala et al., 2001). Oner et al. (2009) reported that biochemical parameters assessed in fish may be a useful tool by providing quantitative measurement of metals impact as well as valuable information of ecological relevance on the effects of metals (Oner et al., 2009). Moreover, biochemical biomarkers are frequently used for detecting or diagnosing sublethal effects in fish exposed to toxic substances (Toguyeni et al., 1997). Sublethal effects are biochemical in origin as the most toxicants exert their effects at basic level of the organism by reacting with enzymes or metabolites and other functional components of the cell. Such effects might lead to irreversible and detrimental disturbances of integrated functions such as behavior, growth, reproduction and survival (Waldichuk, 1979).

As the blood is a pathophysiological indicator of the whole body, biochemical alterations in blood parameters in response to metal exposures may provide important information about the general physiology and health status of the organism as well as water quality (Firat and Kargin, 2010). Blood parameters are increasingly used as indicators of chronic stress status and physiological or sublethal stress responses in fish to endogenous or exogenous changes posing environmental risk (Cataldi et al., 1998; Datta et al., 2007). Barcellos et al. (2004) suggested that alteration of blood biochemistry may be indicative of unsuitable environmental conditions (temperature, pH, oxygen concentration) or the presence of stressing factors, such as toxic chemicals. It is known that physiologic and biochemical parameters in fish blood and tissues can change when exposed to heavy metals (Cicik and Engin, 2005).
Analysis of chemical substances in tissues and body fluids, toxic metabolites, enzymes activities and other biochemical variables have frequently been used in documenting the toxin interaction with biological systems. Plasma and serum reflect the physiologic state of an animal because they are the products of intermediate metabolism (Artacho et al., 2007). The levels of glucose, serum protein, albumin, globulin and activity of enzyme in blood plasma are considered to be specific indicators of sympathetic activation under stress conditions (Lermen et al., 2004; Velisek et al., 2009). Levels of blood or plasma ions and enzymes with important metabolic functions generally indicate the health status of fish (Hrubec and Smith, 1999).

Components like carbohydrate, protein and lipid play a vital role as energy precursors for fish under stress conditions (Umminger, 1970). Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to chemical energy (ATP), which in turn can be expressed as mechanical energy (Lucas, 1996). Changes in carbohydrate metabolism measured as plasma glucose (energy substrate whose production is thought to metabolically assist the animal to cope with an increased energy demand caused by stress) used as general stress indicators in fish (Teles et al., 2007). Glucose (or glucose 6-phosphate) is released through the degradation of glycogen by glycogen phosphorylase (GP) (Roach et al., 1998), and energy is mainly supplied by the oxidation of glucose and lactate as a result of carbohydrate metabolism (Morgan et al., 1997). The glucose concentration was proposed to be mediated by endocrine release such as cortisol (Hontela et al., 1996). Silbergeld (1974) stated that assay of this important blood parameter can serve as an indicator of environmental stress.

Glucose is one of the most sensitive indices of an organism’s stress: Its high concentrations in blood indicate that the fish is under stress and intensively using energy reserves, i.e., glycogen in liver and muscles (Vosyliene 1999; Shalaby, 2007). Glycemia is also one of the classic plasma indicators of stress in fish (Roche and Bogé, 1996). Plasma glucose, liver and muscle glycogen responses appear particularly suitable for measuring stressful levels of pollutants and have long been used as indicators of stress in fish (Pickering et al., 1982; Ramesh, 2001). The plasma glucose concentration in circulation is a function of its production versus absorption by tissues.
In a stress situation, glucose production provides energy substrates to tissues, in order to cope with the increased energy demand. Regardless of the wide use of glucose as an indicator of stress, some authors (Mommsen et al., 1999) emphasized that care has to be taken when using plasma glucose as the only indicator. It has been reported that glucose content is a less precise indicator of stress than cortisol (Pottinger, 1998).

The storage or mobilization of metabolic substrates such as glucose, glycogen, lactate, lipid, and protein are disrupted by exposure to several trace metals, including cadmium (Fabbri et al., 2003), manganese (Barnhoorn et al., 1999), nickel (Sreedevi et al., 1992), and metal mixtures in a polluted habitat (Levesque et al., 2002). Many investigators have reported blood glucose levels under various toxicant exposure conditions; cadmium in Oncorhynchus mykiss, Salmo salar Ctenopharyngodon idellus Cyprinus carpio (Soenges et al., 1996; Joshi and Bose, 2002; Drastichova et al., 2004), copper in Oncorhynchus mykiss (Dethloff et al., 1999); endosulfan in Salmo salar (Petri et al., 2006) and cyfluthrin in Cyprinus carpio (Sepici-Dincel et al., 2009).

Proteins are important organic substance required by organisms in tissue building. They are intimately related with almost all physiological processes, which maintain a simple biochemical system in ‘living condition’ (Joshi and Kulkarni, 2011). Proteins are mainly involved in the architecture of the cell. Proteins occupy a unique position in the metabolism of cell because of the proteinaceous nature of all the enzymes which mediate at various metabolic pathways. During stress conditions fish need more energy to detoxify the toxicant and to overcome stress. Since fish have fewer amounts of carbohydrates so next alternative source of energy is protein to meet the increased energy demand (Singh et al., 2010). Plasma proteins which include globulins, fibrinogens and albumins, serve as a vital function in carrying materials from one part of the fish to another via circulation. They have nutritive, transporting, protective, buffering and energetic functions. The levels of total protein, triglyceride and cholesterol are considered to be major indices of the health status of teleosts. Both the protein degradation and synthesis are sensitive over a wide range of conditions and show changes to a variety of physical and chemical modulators.
Assessment of protein and enzymes activities can be considered as a diagnostic tool to determine the physiological status of cells or tissues (Manoj, 1999). The effects of toxicants on protein content of fish have been observed by a number of investigators; Jung et al. (2003) in Paralichthys olivaceus after exposed to formalin; Jee et al. (2005) in Sebastes schlegeli after cypermethrin exposure; Datta et al. (2007) in Clarias batrachus after exposure to arsenic; El-Sayed et al. (2007) in Oreochromis niloticus after deltamethrin exposure; Min and Kang (2008) in Oreochromis niloticus after benomyl exposure and Kopp et al. (2011) in Cyprinus carpio exposed to microcystins.

Bilirubin is the main bile pigment formed in the liver from the breakdown of heme in red blood cells and other porphyrin rings and can be logically expected to increase proportionally with an increasing level of haemoglobin (Grant et al., 1987; Gupta and Guha, 2006). High values of bilirubin indicate chronic haemolytic disease, hepato cellular disease or bile duct congestion (Burtis and Ashwood, 1996). Serum bilirubin is often used for the evaluations of the liver condition (Jung et al., 2003). Several investigators reviewed the bilirubin concentrations under toxicity conditions. Phosphamidon toxicity to Tilapia mossambica (Jayantha Rao et al., 1984), carbaryl and phorate toxicity to Clarias batrachus (Jyothi and Narayan, 1999), formalin toxicity to Paralichthys olivaceus (Jung et al., 2003), microcystin toxicity to Heteropneustes fossilis (Gupta and Guha, 2006) and glyphosate and zinc sulphate toxicity to Clarias albopunctatus (Okonkwo and Ejike, 2011).

Enzyme activities are considered as sensitive biochemical indicators before hazardous effects occur in fish and are important parameters for testing water and the presence of toxicants (El-Demerdash and Elagamy, 1999; Gül et al., 2004). Such a biochemical approach has been advocated to provide an early warning of potentially damaging changes in stressed fish (Casillas et al., 1983). Enzymatic activities also provide quick screening methods for assessing the health of fish and can be used to determine the incipient lethal concentration of a toxicant. Some of the enzymes are perceived good bioindicators for animals chronically exposed to contaminants such as metals and other xenobiotics (Mazorra et al., 2002).
Phosphatases are important and critical enzymes in biological processes; they are responsible for detoxification, metabolism and biosynthesis of energetic macromolecules for different essential functions (Yousef et al., 2007). Any interference in these enzymes leads to biochemical impairment and lesions of the tissue and cellular function (Enan et al., 1982; Khan et al., 2001). Therefore, by estimating enzyme activities in an organism, we can easily identify a disturbance in its metabolism. Many chemicals at relatively low doses affect the metabolism of biota by altering healthy enzyme activity (Hochster et al., 1972). The responses of various xenobiotic metabolizing enzymes in the fish model are rapidly evolving as important biomarkers for monitoring unacceptable levels of environmental contaminants such as GST, acetyl choline esterase and acid and alkaline phosphatase (Labrot et al., 1996).

Acid (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) (ACP and ALP, respectively) catalyse the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at acid and alkaline pHs, respectively. Acid and alkaline phosphatases are known to be involved in a number of cellular functions such as synthesis, transport, and metabolic regulation such as molecule permeability, detoxification, carbohydrate metabolism, protein synthesis, growth and cell differentiation synthesis of certain enzymes, secretory activity, and transport to phosphorylated intermediates across the cell membranes, steroidogenesis and the biosynthesis of energetic macromolecules for various essential functions. Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Cajaraville et al., 2000), whilst alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Alkaline and acid phosphatase (ALP, ACP) are considered useful biomarkers to determine the pollution level (Basaglia, 2000) and used as a biomarker for a number of diseases (Samman et al., 1996). Induction of these biomarkers is a good approach to measure potential impacts of pollutants on environmental organisms (El-Shehawi et al., 2007).

ACP is hydrolytic lysosomal enzymes released by the lysosomes for the hydrolysis of foreign material; hence it has a role in certain detoxification functions. As the classic macrophage lysosomal marker ACP plays an important part in cellular metabolism, catalyzing hydrolyses of phosphoproteins and the transfer of phosphate
groups (Press et al., 1990). ACP catalyzes the hydrolysis of various phosphate-containing compounds and acts as transphosphorylase in acidic conditions. ACP helps in the autolysis of the cell after its death. It also functions as marker enzyme for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Rajalakshmi and Mohandas, 2005). As well, ACP enzyme can be taken as a reliable tool for the biological assessment of metal pollution (Rajalakshmi and Mohandas, 2005).

Alkaline phosphatases (ALP) form a large family of orthophosphoric monoester phosphohydrolases that non-specifically hydrolyze various mono-phosphates at alkaline pH in vitro (McComb et al., 1979). It is important for hydrolyzing phosphate-containing compound (Lan et al., 1995). Alkaline phosphatases are homodimeric metallo enzymes, and each active site has two Zn ions (Zn1 and Zn2), as well as one Mg ion, which are necessary for enzymatic activity and demonstrates stressor specific responses (Kim and Wyckoff, 1991). Alkaline phosphatases are widely distributed in species ranging from bacteria to human (Backer et al., 2002; Plisova et al., 2005). ALP is a well-known multifunctional membrane enzyme, this enzyme distributed in serum and some other tissues such as liver, bone, kidney and intestine (Bahr and Wilkinson, 1967). ALP is a phosphomonoesterase that detoxifies contaminants during normal living conditions (Zhang, 2004). This enzyme has an important role in mineralization of the skeleton of aquatic animals, bone formation, and its concentration in bone and plasma can be considered as an indicator for bone development (Sakamoto and Yone, 1980; Bernet et al., 2001). The measurement of alkaline phosphatase activity is generally carried out in clinical and ecotoxicological studies. In ecotoxicology, this enzyme may serve as an indicator of intoxication because of its sensitivity to metallic salts (Boge et al., 1988). Therefore, monitoring of these enzymatic activities in tissues and serum for changes due to stress-related homeostatic adjustments can be used for early warning of stress in fish.

ACP and ALP enzymatic activities have been studied in several organisms and the influence of heavy metals has been reported. Many authors observed a series of phosphatases (ACP and ALP) enzyme responses in different piscine systems when exposed to pollutants. Jiraungkoorskul et al. (2003) in nile tilapia, (Oreochromis
niloticus) after glyphosate herbicide exposure; Gill et al. (1990) in rosy barb, (Puntius conchonius) were exposed to mercuric chloride; Molina et al. (2005) and Atencio et al. (2008) in tilapia fish, Oreochromis sp. exposed to microcystins. In fish, Cd can exert a wide range of changes in some plasma stress parameters (i.e. cortisol and glucose) (Fu et al., 1990; Chowdhury et al., 2004) and alterations in the activity of many important enzymes including acid and alkaline phosphatase (Vaglio and Landriscina, 1999; Lionetto et al., 2000). Current accepted opinion of cadmium action as well as other metals is related mainly to their influence on protein molecules, particularly enzymes. They have a strong affinity to bond with the amino acid moieties of proteins and may cause changes in enzyme structures. The most obvious consequences of these changes are the inhibition of enzymes (Drastichova et al., 2004).

It is hypothesized that elevated cadmium levels may cause some impairment to enzymatic processes in fresh water fish. Therefore, these enzymes activities have the potential for serving both as biochemical indicators of toxic stress and sensitive parameters for testing water for the presence of toxicants. With regard to the above explanations, the purpose of this study was to investigate the effect of different concentrations of cadmium on biochemical parameters (glucose, protein, bilirubin, ACP and ALP) in freshwater fish, Cirrhinus mrigala. Further, with the goal of evaluating the possible usefulness of these biochemical parameters as effective environmental indicators for Cd exposure in C. mrigala.
MATERIAL AND METHODS

BIOCHEMICAL PROFILES

The levels of glucose, protein, total bilirubin, acid phosphatase (ACP) and alkaline phosphatase (ALP) were measured in the plasma of fish, *Cirrhinus mrigala*.

ANALYSIS OF PLASMA GLUCOSE

Plasma glucose was assayed with O-Toluidine method (Cooper and Mc Daniel, 1970).

PRINCIPLE

Glucose reacts with O-Toluidine in presence of acetic acid to form a green colour derivative which is measured at 630 nm by using UV spectrophotometer.

REAGENT UTILIZED

Reagent 1 : O-Toluidine colour reagent
Reagent 2 : Glucose standard, 100 mg%

ASSAY PROCEDURE

Four test tubes were taken and marked as Blank (B), Control (C), Test (T) and Standard (S). To each test tube 5 ml of Reagent-1 was added. Then, 0.1 ml of distilled water was added to the Blank (B) test tube. At the same time, 0.1 ml of plasma from control and Cd treated fish were added to the respective tubes. Then, 0.1 ml of Reagent -2 was added to the Standard (S) test tube. The contents in all the tubes were mixed well and heated in boiling water for 10 minutes. Finally, the test tubes were cooled down under running tap water for 5 minutes and the optical density of the test samples were measured at 630 nm within 30 minutes against blank using UV spectrophotometer.
CALCULATION

\[
\text{Plasma glucose in mg/100 ml} = \frac{\text{O.D. of the Test}}{\text{O.D. of the Standard}} \times 100
\]

ANALYSIS OF PLASMA PROTEIN

Plasma protein was measured as described by Lowry et al. (1951).

PRINCIPLE

The final blue color of protein is produced by the reaction of carbamyl groups of protein molecules in the sample with alkaline copper and potassium of the reagent. This complex together with tyrosine and tryptophan of the sample is produced with phosphomolybdate of the Folin phenol reagent.

REAGENTS

Solution A

2.00 gm of sodium carbonate was dissolved in 100.00 ml of 0.1N NaOH.

Solution B

500.00 mg of copper sulphate was dissolved in 100.00 ml of 1% sodium potassium tartarate solution.

Solution C

50.00 ml of solution A was mixed with 1 ml of solution – B.

Folin – phenol reagent

1.0 ml of folin – phenol reagent was mixed with 1.0 ml of double distilled water.
ASSAY PROCEDURE

Four test tubes were taken and marked as Blank (B), Test (T), Control (C) and Standard (S). 1 ml of distilled water was added to ‘Blank’ tube. Then, 0.10 ml of plasma from control and Cd treated fish were taken in respective tubes (Test and Control tubes). In addition, 0.90 ml of distilled water was added. Then, Blank, Test and Control tubes were treated with 5.0 ml of Solution – C for 10 minutes and 0.5 ml of Folin-phenol reagent was added. The color intensity (O.D) of ‘Control’ (C) and ‘Test’ (T) against ‘Blank’ (B) was read after 15 min at 720 nm by using UV spectrophotometer.

For the preparation of ‘Standard’(S) 1.0 mg of bovine serum albumin (Sigma-Chemical company, USA) was added to 10.0 ml of IN NaOH and made up to 100.0 ml in a solution standard flask. From this, 1.0 ml of solution was taken in ‘Standard’ tube and mixed with 0.5 ml of Solution -C, kept for 10 min, then 0.5 ml of Folin phenol reagent was added. The optical density of the ‘Standard’ (S) was read as mentioned above.

CALCULATION

\[
\frac{\text{OD of Unknown}}{\text{OD of Known}} \times \text{Concentration of Standard} = \mu\text{g of protein in ml of plasma.}
\]

ANALYSIS OF TOTAL BILIRUBIN

Using commercially available kit by Span Diagnostics Ltd., the level of plasma bilirubin was estimated by Malloy and Evelyn (1937).

PRINCIPLE

Bilirubin couples with diazotized sulfanilic acid, forming azobilirubin, a red-purple colored product in acidic medium.
Bilirubin + diazotized sulfanilic acid  \[ \xrightarrow{\text{H}^+} \]  azobilirubin  
\[ \downarrow \text{H}^+ \]  Red – purple color  
(\( \lambda_{\text{max}} : 540 \text{ nm} \))

**REAGENTS**

- Reagent 1 : Diazo – A
- Reagent 2 : Diazo – B
- Reagent 3 : Diazo blank
- Reagent 4 : Methanol
- Reagent 5 : Artificial standard (\( \equiv 10 \text{mg}\% \text{bilirubin} \))

**Preparation of working solution**

Diazo reagent: 1 ml of reagent 1 mixed with 0.030 ml of reagent 2

**ASSAY PROCEDURE**

For plasma bilirubin estimation, four test tubes were taken and marked as T1 and T2 for Control and T1 and T2 for Test. 0.05 ml of plasma was added to both the T1 and T2 tubes followed by 0.45 ml of distilled water. Then, Reagent 3 was added to the T2 tube alone. After, diazo reagent was added to T1 tube alone. Finally, 0.625 ml of Reagent 4 was added to both the T1 and T2 tubes, respectively. The tubes were mixed well and kept in dark for 30 min. at room temperature and read the O.D. against distilled water at 540 nm on a spectrophotometer. For standard, Reagent 5 [artificial standard (\( \equiv 10 \text{mg}\% \text{bilirubin} \))] was measured as such against distilled water on a spectrophotometer.

**CALCULATION**

\[
\text{Total (A) = O.D. of T1 - O.D. of T2} \\
\text{Total bilirubin = \( \frac{\text{O.D. of Standard}}{\text{OD of Standard}} \) X 10 = mg/100 ml of plasma}
\]
ANALYSIS OF PLASMA ACID PHOSPHATASE (ACP)

Using commercially available kit by Aspen Laboratories, the activity of plasma total acid phosphatase (ACP) was estimated as directed by Tietz (1976).

PRINCIPLE

The enzymatic reaction sequence employed in the assay of acid phosphatase is as follows:

\[ \alpha - \text{Naphthylphosphate} + \text{H}_2\text{O} \rightarrow \alpha - \text{Naphthol} + \text{Inorganic phosphate} \]
\[ \alpha - \text{Naphthol} + \text{Fat Red TR} \rightarrow \text{Diazido Dye (Chromophore)} \]

The \( \alpha \)-naphthol released from the substrate \( \alpha \)-naphthylphosphate by acid phosphatase is coupled with Fat Red TR to produce a coloured complex which absorbs light at 405 nm. The reaction can be quantitated photometrically because the coupling reaction is instantaneous.

REAGENTS

Acid phosphatase Reagent: \( \alpha \)-naphthyl Phosphate 3mM, Sodium Citrate 60 mM,
\[ \text{pH } 5.3 \pm 0.1 \]

Preparation of acid phosphatase reagent

Reconstitute acid phosphatase reagent with volume of distilled water and swirl to dissolve.

ASSAY PROCEDURE

For the estimation of plasma ACP, two test tubes were taken and marked as Test (T) and Control (C). To the test tubes 1000 \( \mu \)l of acid phosphatase reagent was added. Then, 100\( \mu \)l of plasma from control and cadmium treated fish was dropped in respective tubes. The content in all test tubes were mixed well and incubated at 37\( ^{\circ} \) C
for five minutes. The optical density was read at one minute interval and five additional absorbance were taken against purified water at 405 nm using UV spectrophotometer.

**CALCULATION**

\[
\text{Total acid phosphatase} = \frac{\Delta A / \text{Min.} \times 10^6 \times 1.1}{12.9 \times 10^3 \times 1.0 \times 0.1} = \Delta A / \text{Min.} \times 853 = \text{U/L}
\]

**ANALYSIS OF PLASMA ALKALINE PHOSPHATASE (ALP)**

Using commercially available kit by Span Diagnostics Ltd., the activity of plasma alkaline phosphatase (ALP) was estimated by Kind and King (1954).

**PRINCIPLE**

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-Aminoantipyrine in presence of the oxidising agent Potassium Ferricyanide and forms an Orange-Red coloured complex, which can be measured calorimetrically. The colour intensity is proportional to the enzyme.

The reaction can be represented as:

\[
\text{Phenyl Phosphate} \xrightarrow{\text{Alkaline phosphatase}} \text{Phenol + Phosphate}\]

\[
\text{Phenol + 4-Aminoantipyrene} \xrightarrow{\text{Potassium ferricyanide}} \text{Orange-red complex.}
\]

**REAGENTS**

- Reagent 1 : Buffered Substrate, pH 10.0
- Reagent 2 : Chromogen Reagent
- Reagent 3 : Phenol Standard, 10 mg%
Preparation of working reagent solution

Reconstitute one vial of Reagent-1 (Buffered substrate) with 2.2 ml of purified water.

ASSAY PROCEDURE

For the estimation of plasma ALP, four test tubes were taken and marked a Blank (B), Standard (S), Control(C) and Test (T). 0.5 ml of Reagent-1 was added to all the test tube. In addition, 1.5 ml of purified water was added. The tubes were mixed well and incubate at 37º C for 3 minutes. Then, 0.05 ml of plasma was added to the Test (T). Then, 0.05 ml of Reagent-3 was added to the Standard (S). The tubes were mixed thoroughly and incubate at 37º C for 15 minutes. Then, Reagent-2 was added to all the tubes. Then, 0.05 ml of plasma was added to the Control (C). The O.D of Blank (B), Standard (S), Control (C) and Test (T) was measured using UV spectrophotometer against purified water at 510 nm.

CALCULATION

\[
\text{Plasma alkaline phosphatase activity (in KA units) = } \frac{\text{O. D. of Test} - \text{O. D. of Control}}{\text{O. D. of Standard} - \text{O. D. of Blank}} \times 10
\]
RESULTS

Cadmium-induced biochemical responses in plasma glucose, protein, total bilirubin, ACP and ALP, after acute exposure to cadmium was shown in Table 16 and Fig. 15a, b, c, d & e. Plasma glucose and bilirubin level was found to be increased in cadmium treated fish showing a percent increase of 69.00 and 27.77 at the end of 24 h respectively. On the other hand, plasma protein, ACP and ALP levels were decreased in cadmium exposed fish showing a percent reduction of 56.13, 43.57 and 32.49 respectively. The values indicates significant difference from control (P< 0.05; except bilirubin and ACP activity).

Changes in plasma glucose level of fish *Cirrhinus mrigala* exposed to sublethal concentration of cadmium was presented in Table 17 and Fig. 16. In Treatment I, plasma glucose level was found to be increased throughout the exposure period. However, on day 28th a significant decrease in plasma glucose was noted (1.49). Whereas, plasma glucose level was found to be increased from 7th to 35th day in Treatment II. A maximum increase of 99.21 % was noted at the end of 35th day of exposure. In comparison, Treatment II showed the maximum elevation. There were significant (P<0.05) variation among the treatments (F2, 60 = 241.77; P<0.05), significant among periods (F4, 60 = 15.48; P<0.05) and their interactions (F8, 60 = 51.28; P<0.05).

Table 18 and Fig. 17 shows the decreased level of plasma protein of fish *Cirrhinus mrigala* exposed to sublethal concentration of cadmium for 35 days. In Treatment I a maximum percent decrease of 75.71 was noted at the end of 7th day. Whereas in Treatment II, a maximum percent decrease of 61.37 was noted at the end of 35th day. When comparing the treatments, Treatment I (3.59 mg L⁻¹) showed a maximum reduction. There were significant (P<0.05) variation among the treatments (F2, 60 = 29.80; P<0.05), not significant among periods (F4, 60 = 1.54; P>0.05) and their interactions (F8, 60 = 9.54; P <0.05).

Plasma bilirubin level of *C. mrigala* exposed to sublethal concentrations of cadmium was shown in Table 19 and Fig. 18. Both the treatments showed increased
level of bilirubin as the exposure prolonged. A maximum increase in plasma bilirubin level was noted at the end of 35th day in both the treatments showing 80.82 and 98.56 for Treatment I and II respectively. In comparison, Treatment II showed maximum increase of bilirubin. There were significant (P< 0.05) variation among the treatments (F2, 60 = 70.23; P<0.05), not significant among periods (F4, 60 = < 1; P >0.05) and their interactions (F8, 60 = 4.00; P <0.05).

Changes in plasma ACP activity of fish *Cirrhinus mrigala* exposed to sublethal concentrations of cadmium and their percent variations in comparison to controls are presented in Table 20 and Fig. 19. ACP activity of Treatment I showed a decreasing trend as the exposure progressed. In Treatment II the ACP activity was found to be decreased up to 21st day. However, on day 28th and 35th day, a significant increase in ACP activity was noted showing a percent increase of 12.15 and 22.51, respectively. When comparing treatments Treatment II showed highest variations. There were significant (P<0.05) variation among the treatments (F2, 60 = 85.76; P< 0.05), significant among periods (F4, 60 = 21.22; P<0.05) and their interactions (F8, 60 = 13.11; P< 0.05).

Alterations in plasma ALP activity of fish exposed to sublethal concentrations of cadmium was shown in Table 21 and Fig. 20. The mean values of ALP activity was found to be 5.09 ± 0.77, 6.94 ± 1.00, 5.85 ± 0.75, 6.26 ± 0.97 and 6.23 ± 1.26 in KA units for Treatment I and 4.96 ± 1.02, 7.33 ± 1.50, 5.20 ± 1.13, 6.17 ± 1.08 and 6.82 ± 0.70 in KA units, for Treatment II at the end of 7, 14, 21, 28 and 35th day, respectively. A maximum percent increase of 81.25 and 98.42 (Treatments I and II) was noted at the end of 35th day of exposure. In comparison, Treatment II showed highest elevations. There were significant (P<0.05) variation among the treatments (F2, 60 = 5.98; P<0.05), not significant among periods (F4, 60 = 1.86; P>0.05) and their interactions (F8, 60 = < 1; P >0.05).
DISCUSSION

Higher concentrations of toxicants in aquatic environment cause adverse effect on aquatic organisms at cellular or molecular level and ultimately it leads to disorder in biochemical composition. Numerous biochemical indices of stress have been proposed to assess the health of non-target organisms exposed to toxic chemicals in aquatic ecosystem (Nimmi, 1990). Any environmental disturbance can be considered a potential source of stress, and theoretically can be detected by changes in the plasma substrate concentrations, or by changes in erythrocytes parameters such as cell volume and enzyme activities. Other blood-biochemical parameters of plasma, including levels and activities of the organic and inorganic enzymes are generally believed to be good stress indicators and are influenced by diverse factors in fish (Roche and Bogé, 1995).

In polluted areas, exposure of fish to heavy metal leads to interactions between these chemicals and biological systems, which give rise to biochemical disturbances (Talas et al., 2008). Fish are responding to various stressors by a series of biochemical and physiological stress reactions, so called secondary stress responses comparable to those of higher vertebrates (Mazeaud and Mazeaud, 1981). An increase in glucose concentration is a secondary response to stress, and the level of the increase is a measurement of stress response which has been widely used in a variety of fish species (Barton, 2000). Earlier reports on the changes in blood glucose in response to stress are contradictory showing both a rise (Yildiz and Pulatsu, 1999; Mzimela et al., 2002) and a fall (Krumshnabale and Lackner, 1993; Flodmark et al., 2002).

Glucose concentration is influenced by environmental and metabolic factors and will generally increase under stress (Bahmani et al., 2001; Bayir et al., 2007). Metals can induce hyperglycaemia in different fish species (Varanka et al., 2001; Zikic et al., 2001). Similar observations have also been reported by in fish treated with the metal cadmium (Fu et al., 1990; Hontela et al., 1996). An increase in blood glucose is common in animals encountering a stressful situation, and it is one of the major effects of the secretion of catecholamine and corticosteroid hormones that
occurs in such situations (Brown, 1993). Stress-related hyperglycemia in fish is thought to be mediated by cortisol and epinephrine as an increase in these hormones usually precedes hyperglycemia. Similarly, Menton (1927) reported increase in the circulating glucose levels is also related to increases in the concentration of circulating catecholamines or corticosteroids following stress or increased plasma glucose levels due to stress may also be a response to respiratory insufficiency. Wedemeyer and McLeay (1981) stated that high levels of blood glucose are caused by disorders in carbohydrate metabolism appearing in the condition of physical and chemical stresses.

A variety of stressors stimulate the adrenal tissue, resulting in increased level of circulating glucocorticoids (Hontela et al., 1996; Richard et al., 1998) and catecholamines (Mazeaud and Mazeaud, 1981; Witters et al., 1991). Both of these groups of hormones produce hyperglycaemic. The present study sustains with the above findings stated that both acute as well as sub lethal exposure, exhibits hyperglycaemic condition of glucose. It may be due to alteration in the carbohydrate metabolism due to cadmium induced stress. Significant increase in plasma glucose level has also been reported in fish exposed to cadmium (Almeida et al., 2001; Wu et al., 2007). Wedemeyer and Yasutake (1977) reported that initial increase in glucose level was due to glycogenolysis, but a sudden reduction can be correlated to utilization of stored glycogen in order to meet the energy requirements of the fish. A decrease of glucose is linked to depletion of reserve energy. In the present study also decreased glucose content observed at the end of 28th day of sublethal exposure (Treatment I), may be attributed to hypoglycaemia resulted from utilization of stored glycogen in order to meet the energy requirements of the fish. Reductions in the blood glucose level were observed in Clarias batrachus exposed to carbaryl (Sharma, 1999), and in S. mossambicus exposed to ziram (Nivedhitha et al., 1998).

The protein content of the cell is considered to be an important tool for the evaluation of physiological standards. They are central to several vital blood activities, including homeostasis and blood coagulation, vitamin and hormone transport, and specific immunity to pathogens (Leatherland et al., 1998). Total protein is additionally used as an indicator of liver impairment. The adverse effect of pollutants on the serum total protein (TP) levels in fishes has been studied by many researchers (McKim et al.,
1970; Gluth and Hanke, 1984). They found that the decrease in TP in fish exposed to toxic levels of toxicants could be attributed to either a state of hydration and change in water equilibrium or a disturbance in liver protein synthesis, or both. Nayak et al. (2004) reported that the reduction of total serum protein content during deltamethrin-toxicity may indicator not only of hepatic dysfunction, but also of immunosuppressive effect of the deltamethrin. Decreased protein levels after cypermethrin exposure may be attributed to stress-mediated mobilization of these compounds to fulfill an increased demand for energy by the fish to cope with detrimental conditions imposed by the toxicant (Jee et al., 2005).

Another possibility for the protein reduction may be due to the blocking of protein synthesis or protein denaturation or interruption in the amino acid synthesis (Jha, 1991). Moreover, the chemical exposed animals obtain extra energy requirement from the tissue protein (Yadav et al., 2007). The reduction in protein content indicates that the tissue protein may undergo proteolysis, which results in the production of free amino acids and is used in the trichloroacetic acid cycle for energy production under stress conditions. Fish under stress also mobilize protein to meet energy requirements needed to sustain increased physical activity (Sievers et al., 1995). To overcome the stress, the animals require high energy. This energy demand might have led to the stimulation of protein catabolism (Sancho et al., 1997).

The decline in protein could be attributed to the fact that heavy metals in general interfere with protein synthesis (Syversen, 1987). Also the utilization of protein in cell repair and organization cause the depletion in the tissues, and the metal-induced change may probably affect the enzyme-mediated biodefense mechanism of the fish (Vutukuru, 2003). Bradbury et al. (1987) pointed out that the decreased protein content might also be attributed to the destruction or necrosis of cellular function and consequent impairment in protein synthesis. The present study gives a support to the above findings and decreased protein level during study may be due to cadmium induced dysfunction in tissues or to compensate for an increased demand for energy or due to the disruption of protein synthesis or denaturation of protein or interruption in the amino acid synthesis.
Changes in serum bilirubin levels were also included to investigate the degree of liver damages (Min and Kang, 2008). A common response in fish exposed to aquatic toxicants is a reduction in RBC counts and/or an increase in the proportion of immature RBC (Panduranga Rao et al., 1990): this situation is frequently associated with an increase in serum bilirubin concentration (Andersson et al., 1988). Datta et al. (2007) reported that hyperbilirubinemia in arsenic exposed *Clarias batrachus* might have resulted from either due to hemolysis or due to irregularities in the uptake and conjugation of bilirubin by the liver cells. Increase in bilirubin concentration was reported by Carbis et al. (1996) and Young et al. (1994) in treated fishes. The higher bilirubin value in cultured *Channa argus* was a consequence of the increased level of haemoglobin (Gul et al., 2011).

According to Pepeljnjak et al. (2003), toxic substances increase values of bilirubin in the common carp and higher values of bilirubin may indicate hepatic injury. Hyperbilirubinemia suggests either liver damage or obstruction of the bile ducts. In some forms of anaemia, particularly of haemolytic type, the (bilirubin) level may rise because of the inability of the liver to eliminate the increased quantity of the pigment. Hyperbilirubinemia is also a characteristic of obstructive jaundice. Thus, whatever the cause, the elevated level of serum bilirubin suggests malfunction of the liver (Jyothi and Narayan, 1999). Liver damage indicated by elevated serum aspartate aminotransferase and alanine aminotransferase activity could reduce hepatic function, which may be associated with an increase in serum bile acid and bilirubin concentration (West et al., 1987).

In the present study the significant increase in plasma bilirubin during acute and sublethal treatment may be due to hemolysis or irregularities in the uptake and conjugation of bilirubin by the liver cells. The elevation in bilirubin seems to be a phenomenon occurring secondarily because of RBC haemolysis. Hemolytic anemia, a reduction in red blood cells due to destruction, is a possible problem. This usually increases the bilirubin, one of the breakdown products of hemoglobin in the blood, more of a problem in toxicants exposed to fish. The present study corroborates the above findings posited that cadmium exposed *Cirrhinus mrigala* showed increase in bilirubin level may be a cause of severe cellular damage leading to impaired cellular function or inability of the liver to eliminate the toxicant.
It is generally accepted that an increase in enzyme activity in the extra cellular fluid or plasma is a sensitive indicator of even minor cellular damage, since the level of these enzymes are higher than the normal levels in the extra cellular fluids (Van der Osst et al., 2003). Shakoori et al. (1990) reported that the increase of blood enzymatic activity is either due to (1) leakage of these enzymes from hepatic cells and thus raising levels in blood, (2) increased synthesis and (3) enzyme induction of these enzymes. Also, Tietz (1987) and Campbell et al. (1984) reported that these enzymes liberate to the blood stream when the hepatic parenchyma cells are damaged.

Alkaline phosphatase is an intrinsic plasma membrane enzyme of almost all animal cells. ALP changes are due to the accumulation of contaminants in fish (Karuppasamy, 2000). Gupta and Dhillon (1983) studied the effects of sublethal concentrations of xenobiotics in the plasma of Claris batrachus and Cirrhina mrigala to estimate liver damage by measuring plasma ALP as an indicator for liver cell necrosis. ALP is sensitive to metals and the effects of metals on ALP have been reported (Mazorra et al., 2002; Atli and Canli, 2007). Elevated ALP activity is commonly observed as a result of intra- and extra-hepatic obstruction to biliary passage. It was shown that Cu exposures increased ALP activity in the serum and gill in Cyprinus carpio and this increase was correlated with tissue alterations and cell damage (Karan et al., 1998). Serum ALP used as indices of arsenic-induced hepatotoxicity (Min and Kang, 2008). Damage to the liver cell may result in leakage of the enzymes into the serum because of a large concentration gradient. The increase in enzyme activity may be related to the changes in the histological structure of the hepatic and extra-hepatic tissues (El-Sayed et al., 2007). Increase in ALP may be caused by the obstruction of the biliary canal or osteoblast activity and feeding conditions (Moraes et al., 2005). In the present study, the elevation in ALP activity during sublethal treatment may be due to cell necrosis in liver.

Bernet et al. (2001) showed that there was a decrease in serum ALP activity in Salmo trutta exposed to effluent from the sewage treatment comparing to the tap water suggesting that the decrease of ALP activity may result from disturbance of the membrane transport system. Das et al. (2004) reported that reduction in ALP activity in serum, brain and gill, after nitrite toxicity to Catla catla, Labeo rohita and Cirrhinus mrigala. Such reduction of enzyme activity in tissues and serum can
probably be attributed to the decrease in intracellular pH of the substrate (tissues/blood) due to the nitrite buildup in blood and tissues of the exposed fish. A similar mechanism may be operated in the present study during acute exposure indicating a significant decrease in plasma ALP activity. Decrease in ALP activity may be taken as an index of hepatic parenchymal damage and hepatocytic necrosis (Onikienko, 1963). Inhibition of ALP reflects alteration in protein synthesis and uncoupling of oxidative phosphorylation (Verma et al., 1984).

The elevation in ACP activity is probably related to increase in lysosomal activity in the injured cells occurring as part of prenecrotic changes. ACP was higher in fish indicating that phosphatase esters may be hydrolyzed faster. Rao (2006) measured an increase in plasma activity of ACP following exposure to organophosphorus insecticides in a euryhaline fish (*Oreochromis mossambicus*). Elevated ACP activities in serum and brain increases with increased acidic conditions might be attributed to pH reduction in these substrates (tissues/blood) (Das et al., 2004). Bernet et al. (2001) reported that the enhancement of the enzyme can be observed as a signal of tissue damage. ACP activity was high indicating derangement of defense mechanisms or a delay in revival (Rajalakshmi and Mohandas, 2005). In the present study, the significant increase in plasma ACP activity during sublethal treatment of Treatment II is possibly related to increase in lysosomal activity in the injured cells occurring as part of prenecrotic changes.

Decrease in acid phosphatase activity could result in enhanced toxicity of environmental chemicals or endogenous compounds and the possibility that some forms of neoplasms may be induced in fish. Intoxication by mercury caused depletion of energetic resources and metabolic malfunction by inhibition of acid phosphatases in grass carp (Shakoori et al., 1994). It is reported that ACP activities showed a decrease with increasing metal concentrations such as Cd, Cu and Pb (Ozmen et al., 2006). Hinton et al. (1973) have also noted an initial increase and later fall in acid phosphatase activity due to mercury intoxication. The decreased activities of these enzymes indicate disturbance in the structure and integrity of cell organelles, like endoplasmic reticulum and membrane transport system (Nchumbeni et al., 2007). In the present study also the significant decrease in ACP activity during acute treatment may be due to alterations in the structure and integrity of cell organelles due to high
accumulation of cadmium. Although high variances are expected in serum enzyme measurements (Folmar et al., 1992, 1993), variations between blood chemistry parameters among fish species depend on the sampling technique, analysis methods, age, habitat and diet (Celik, 2004).