DISCUSSION

Anthropogenic activities are responsible for increasing levels of pollutants in the aquatic environment all over the world (Zaharia and Suteu, 2012). Complex mixtures of pollutants cause deleterious effects on the quality of water as well as on fish and other aquatic organisms. Fish acts as an important indicator of water quality under such conditions as it remains in direct contact with water for food and oxygen and is highly sensitive to any change in its environment (Kaur et al., 2013). Fish adjusts its metabolism to adapt to the altered environment, therefore biochemical markers such as enzymes, proteins and amino acids of fish have been widely used in the studies related to toxicology, ecotoxicology and pharmacology. Stress induced variation in these biomarkers is not only useful for determination of physiological responses and health status of fish but also provides important information for integrated evaluation of the effect of pollutants on the health of an aquatic ecosystem (Cajaraville et al., 2000; Li et al., 2007).

A dose and duration dependent increase in black color on the body, gills and viscera of the exposed fish indicates absorption/adsorption of present dye by the test fish. Only a slight change in the intensity of black color from the tissues till the end of the recovery period hints towards strong affinity of the dye for the tissues of the fish. Several dyes have been reported to have high affinity for the tissues of fish (Tonogai et al., 1979, 1980; Singh, 2007).

5.1 Antioxidant/detoxification enzymes

Antioxidant/detoxification enzymes are the main effectors of all the transformations occurring in the body of a fish and may produce extensive changes in structural and toxicological properties of contaminants for their complete conversion into innocuous inorganic end products (Rao et al., 2010). Changes in the enzyme activity are therefore considered an early warning of the adverse effects before the onset of serious pathological damage in the exposed animals (Regoli et al., 1998). In the current study, exposure to AB-1 induced a variable oxidative damage in liver, kidney, gill, muscle and brain of L. rohita as there was a tissue specific marked change in the activity of selected enzymes. It was observed that GR, GPx, AcP, GSSG and MDA
increased while SOD, SDH, LDH, ALT and AChE decreased in all the tissues of the fish after 96h as well as 150 days of exposure to the dye. This dye was observed to have a long lasting effect on the defenses of the fish as hardly any reversal in the activity of most of the enzymes, contents of antioxidants, proteins and amino acids as well as the secondary structure of proteins was observed in the selected tissues of the exposed fish even after 90 days of the recovery period.

In the present study, as the test fish tried to overcome the stress of AB-1, the reactions of both phase I and phase II of biotransformation pathways were strongly affected by this dye or its metabolites. Phase I pathway includes oxidative, reductive and hydrolytic reactions. GR is an important antioxidant of phase I which plays a role in cellular protection and adjustment processes of metabolic pathways under stress. It plays a key role in maintaining proper function and preventing oxidative stress in animal cells and can act as a scavenger for hydroxyl radicals, singlet oxygen, and various electrophiles. The induction of GR activity is considered a potential biomarker of oxidative stress in living organisms (Stegeman et al., 1992). Therefore, dose dependent increase in GR activity of all the tissues of the fish after short term and long term exposures as well as both the recovery periods can be directly related to the oxidative stress of present dye on the defense mechanisms that might have caused accumulation of GSH. GR generally increases under such conditions to recycle excess of GSH. Enhanced GR activity in liver of azo dye induced rats has been observed by Oh and Lee (1981). Elia et al. (2006) observed an increase in GR activity in the liver of NaClO exposed carp. During the recovery period, the dye exposed fish tried to adjust to the stress as there was a fluctuation in brain and GR activity declined in liver, kidney and gill. Visweswaran and Krishnamoorthy (2012) also observed fluctuations in GR activity in the testis of Tartrazine treated rats. Increased GR activity in fish has been observed in stressed animals by other workers also (Petrivalsky et al., 1997; Lushchak et al., 2009). In the present study, maximum increase in GR activity was observed in liver after 96h (82.57%) on exposure to 10 mg/l dye whereas increase in liver was even more (88.56%) due to 2.5 mg/l dye after 50 days of exposure. During the subchronic exposure cumulative effect of the dye was more evident as highest increase was observed in muscle (90.21%) on exposure to 2.5 mg/l dye. Inhibition of GR activity in
Discussion

liver, kidney and gill during the recovery period after subchronic exposure could be due to the change in the availability of NADPH in these tissues under the stress of the present dye as suggested by Ballesteros et al. (2009). Bainy et al. (1996) reported decrease in the activity of GR in the liver of Nile tilapia from a polluted site. The decrease in GR considered an indicator of impaired reduction of GSSG to GSH due to the depletion of NADPH, a cosubstrate required for GR (Zhang et al., 2005; Huculeci et al., 2008). In the present study also, the decline in GR corresponded to a rise in GSSG and a decline in GSH in the brain of dye exposed fish.

GPx is involved in the detoxification of ROS, therefore it plays an important role in defense against lipid peroxidation and oxidative damage (Winston and Di Giulio, 1991). A dose and duration dependent increase in GPx activity of the dye exposed fish clearly indicates that the present dye induced ROS generation which was in turn responsible for an increase in the level of the enzyme in its tissues. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Steinberg, 2012). Ballesteros et al. (2009) suggested that the increase of GPx activity could trigger increase in GR activity to maintain the cytosolic concentration of GSH. Similar observation was recorded at various doses of the dye during the 96h exposure and the recovery period following it in the present study. Same trend was observed during the subchronic exposure also but during the recovery period after this exposure the stress overpowered and the trend could not be maintained in the dye exposed fish. Increase in GPx activity after subchronic exposure was ~2 fold higher as compared to the values after 96h exposure but during recovery period there was slight improvement as the increase was ~1.2 fold higher in comparison to the increase after 96h exposure. Highest induction of GPx in the tissues of the dye exposed fish till the end of the recovery periods indicates that the antioxidant pathway that was stimulated by present dye during exposure probably resulted in increased production of peroxides with time. Although this enzyme acts principally in the removal of organic peroxides, it is also involved in the metabolization of H$_2$O$_2$ (Zhang et al., 2004). Pandey et al. (2001) also correlated the increase in GPx activity in the fish exposed to endosulfan with the level of H$_2$O$_2$. Activation of GPx may also be a response to compensate the inhibition of CAT in gill, muscle and brain of the
present fish as suggested by Modesto and Martinez (2010). More increase in GPx till 45 days of the recovery period after both the exposures probably reflects an adaptation to the dye induced oxidative conditions which the fish might have experienced during exposure (Lenartova et al., 1997). Santos et al. (2004) highlighted that various organic and inorganic redox active contaminants had longer influence in exposed animals, this could be the reason for a continuous and higher increase in GPx even due to minute doses of AB-1 (0.625-2.5 mg/l dye) during the subchronic exposure as well as till the end of the recovery period. Decline in GPx activity in kidney after 96h exposure hints towards involvement of the tissue in neutralizing the impact of peroxides formed during acute exposure to the present dye. This decline could be due to corresponding reduced level of glutathione under the stress of AB-1 as suggested by Kaddissi et al. (2012) in crayfish Procambarus clarkia on exposure to uranium. Non-protein thiol plays an important role in ROS scavenging, since it is a substrate for the removal of H$_2$O$_2$ by GPx. The change in GPx activity is generally accompanied by change in the level of GSH, which is the co-substrate for H$_2$O$_2$ decomposition by GPx (Sies, 1999). However, Vijayavel et al. (2004) related the decline in GPx to reduced enzyme synthesis or more O$_2^-$ production in naphthalene exposed S. serrata. The activity of GPx has been reported to decrease in the liver of DAB and BHA treated rats by Oh and Lee (1981). A reduced GPx activity in kidney after 96h exposure indicated that its antioxidant capacity was exceeded by the amount of hydroperoxide products. It also reflected a possible failure of the antioxidant system of the exposed fish (Monteiro et al., 2006). Substrate competition between GPx and CAT is also considered to be the cause of the reduction in GPx (Cheung et al., 2004). In the present study also, reduction in GPx corresponded with elevated CAT in the tissues of the dye exposed fish. Visweswaran and Krishnamoorthy (2012), however, observed fluctuations in GPx activity in the testis of Tartrazine treated rats.

CAT is an effective protective enzyme against lipid peroxidation (Winston and Di Giulio, 1991) therefore dose dependent significant increase in the activity of CAT in liver and kidney throughout the exposures and recovery periods of the present study could have been for protection against lipid hydroperoxides and H$_2$O$_2$ under the stress of AB-1. CAT activity of liver was affected more after 96h exposure while that of
kidney was affected more during subchronic exposure. Almost same increase in CAT activity of the liver due to 10 mg/l dye after 96h and due to 2.5 mg/l dye by 150 days of subchronic exposure clearly indicates the cumulative effect of AB-1 with duration. Atli and Canli (2010) associated stimulation of CAT activity with an effective antioxidant defense system acting against oxidative stress and/or compensating for the decrease in other antioxidant enzymes, such as SOD and GPx. In the present study also, there was a marked decline in SOD in the tissues with an increased CAT activity. An increased CAT activity has also been observed by other workers in the liver of *C. auratus* exposed to cadmium and naphthalene, phenanthrene, 3,3’-dimethylbenzidine, nitrobenzene and textile mill effluent (Shi et al., 2005; Yin et al., 2007; Liu et al., 2009; Peng et al., 2010; Zagal and Mazmanci, 2011) and DAB and BHA treated rats (Oh and Lee, 1981). Superoxide anion dismutation to the CAT substrate H$_2$O$_2$ (Winston and Di Giulio, 1991; Vega-Lopez et al., 2009) may also have accounted for increase in CAT activity in the tissues of the present fish on exposure to AB-1. Decline in the CAT activity in gill, muscle and brain of the present fish during the exposures as well as the recovery periods may have been due to the flux of O$_2$ or insufficient supply of NADPH under the stress of the present dye. Flux of O$_2^-$ and insufficient NADPH under stress has been correlated with an inhibition of CAT activity in the tissues by Kono and Fridovich (1982). Low level of SOD in the present fish clearly indicates low levels of NADPH in its tissues which are required for the activation of CAT from its inactive form. Decline in CAT activity of gill throughout the study could have been due to diffusion of H$_2$O$_2$ in surrounding water (Wilhelm-Filho et al., 1994) thus low concentration of H$_2$O$_2$ may have decreased CAT activity. CAT is considered to be an ineffective scavenger of H$_2$O$_2$ at a low concentration (Dandapat et al., 2000). CAT inhibition has also been related to the accompanied direct binding of metal ions to –SH groups on the enzyme molecule and superoxide radical under an oxidative stress (Atli and Canli, 2010). CAT reduction due to stress induced damage of the CAT active site has also been reported by Bainy et al. (1996). However, Bagnyukova et al. (2006) have suggested that the superoxide anion may be responsible for decreased CAT activity, which could also be possible in the present fish as an increase in GPx in all the tissues on exposure to AB-1 prolonged during the recovery periods. A decline in CAT activity due to dyes has also been
SOD serves to protect cells against the oxidative damage of free radicals by catalyzing the conversion of superoxide anion (O$_2^-$) to molecular oxygen (O$_2$) and hydrogen peroxide (H$_2$O$_2$). In the current study, decrease over control in the activity of SOD in all the tissues after both the exposures and continued decline till the end of recovery periods post exposure, clearly hints towards the prolonged oxidative stress of the present dye. Our results are in good agreement with the findings of Visweswaran and Krishnamoorthy (2012), who observed significantly reduced SOD activity in the testis of Wistar rats after 60 days of oral administration of Tartrazine (E102). A significant reduction in SOD activity has also been observed in C. auratus on exposure to nitrobenzene (Peng et al., 2010) and in C. gariepinus and zebrafish on exposure to textile effluents (Ayoola et al., 2012; Wenjuan et al., 2012, respectively). Reduction in the levels of SOD activity actually seems to be due to the accumulation of superoxide anion radicals under the stress of AB-1. Dimitrova et al. (1994) reported that when produced in excess, the superoxide radicals by themselves or after transformation to H$_2$O$_2$ cause oxidation of the cysteine in the enzyme SOD that leads to deactivation of the enzyme. Baginyukova et al. (2006) have also related excess of H$_2$O$_2$ to reduced SOD activity. SOD is known to be inhibited by its own catalytic activity since the H$_2$O$_2$ produced by the enzyme reduces Cu$^{2+}$ to Cu$^+$ in the active site, and induces a series of fenton reactions. The end products of this cascade (Cu$^{2+}$·OH and its ionized forms) attack the histidine adjacent to the active site through oxidation and inhibit the enzyme (Hodgson and Fridovich, 1975). An increase in superoxide radicals associated with the disruption of antioxidant defenses of fish on exposure to the present dye could also be responsible for reduction in the activity of SOD as suggested by Falfushynska and Stolyar (2009).

A significant decrease over control was observed in the activity of SDH in all the tissues after both the exposures (96h and 150 days) as well as till the end of recovery periods. Sekar et al. (2008) also observed decreased SDH activity in S.
Discussion

*Hydrodroma* exposed to textile dye industry effluent. Decrease in SDH activity has also been reported on exposure of fish to other pollutants like quinolphos and zinc (David *et al.*, 2010; Zheng *et al.*, 2011). In the present study, maximum decline was observed in brain after 96h exposure while muscle was affected more during the subchronic exposure as well as the recovery periods. SDH is an exclusively mitochondrial marker enzyme, located in the inner mitochondrial membrane and is part of both TCA cycle and respiratory electron transfer chain (Rutter *et al.*, 2010). Decline in SDH activity in the current study may be due to an impairment of aerobic respiration as color intensity of dye in all the tissues including gill of the fish increased dose and duration dependently and there was only a slight decline in the intensity of color during the recovery period. At the same time increase in mucus secretion in the dye exposed fish might have reduced the respiratory surface and supply of oxygen. Dose dependent decrease in the SDH activity under the stress of present dye suggests that the aerobic oxidation through the TCA cycle was adversely affected as this decline was present in all the tissues and prolonged throughout the recovery period also. This gets support from the report of Tripathi and Shasmal (2011) who observed that corresponding to a decline in SDH, aerobic utilization of lactate by the tissues was impaired on exposure of the fish to quinolphos.

Decrease in LDH activity in all the tissues in the current study during the exposures as well as the recovery periods could be due to dye induced decrease in biosynthetic activities. It was observed that on exposure to the present dye the test fish swam vigorously for some time but hardly swam thereafter and ate very less during the subchronic exposure as well as the recovery periods. LDH is the terminal enzyme of anaerobic glycolysis, and therefore is of crucial importance to muscle physiology, particularly in conditions of chemical stress when high levels of energy may be required for a short period of time (Monteiro *et al.*, 2007). Decline over control in LDH activity during the exposures as well as till the end of the recovery periods could be due to formation of an enzyme-inhibitor complex that led to impairment of carbohydrate metabolism (Copper and Somero, 1990; Sharma and Gopal, 1995) or reduction in de novo synthesis of proteins (enzymes) in all the tissues of the dye exposed fish. LDH converts lactate to pyruvate and has a very important role in carbohydrate metabolism,
decrease in LDH activity in the current study can be directly correlated to the lethargy shown by the dye exposed fish throughout the subchronic exposure and the recovery periods. Sastry and Siddiqui (1983) reported that decrease in LDH activity reflected a possible decrease in glycolytic capacity of the tissues. Gravato et al. (2010) also reported that reduced LDH activity suggested alterations in the pathways of energy production and a decreased capability of getting energy in response to a sudden stimulus. Valarmathi and Azariah (2002) suggested that stress induced changes in the conformation of active site led to a decline in the activity of LDH. Reduction in LDH activity in liver, gill, muscle and brain of chlorpyrifos exposed *Heteropneustes fossilis* has been reported by Tripathi and Shasmal (2011). They suggested that binding of the pesticide or its metabolites with the enzyme molecule was responsible for decline in LDH activity. Decrease in LDH activity has also been observed in *Pomatoschistus microps* and *C. gariepinus* on exposure to chemicals from estuaries of the Portuguese Northwest coast and lead nitrate, respectively (Monteiro et al., 2007; Osman et al., 2007).

Transaminases are mitochondrial and cytosolic enzymes, involved in the catabolism of amino acids and an increase in transaminase activity is suggested to be directly correlated to mitochondrial disruption and tissue damage which may result in the release of enzymes into plasma (Raju and Ramana Rao, 1985; Tilak et al., 2005). In the current study, an increase in AST activity in liver, kidney and gill while a decrease in ALT activity in all the tissues except for brain of the fish after 96h exposure along with an increase in both AST and ALT activities in all the tissues during subchronic exposure indicates an increase in biotransformation and detoxification processes under the stress of the present dye. This increase might have resulted due to the AB-1 induced tissue and mitochondrial damage. Increased ALT has been suggested to be an indicator of the damage to the integrity of hepatocyte membrane by Mitchell et al. (1980) and the elevated AST activity is considered to be due to mitochondrial disruption (Schmidt and Schmidt, 1974). Elevation in transaminases is also used to determine liver damage as it indicates the utilization of amino acids for the oxidation or for glucogenesis (Philip et al., 1995). Besides, the glutamate would also be used for the synthesis of glutathione which goes a long way in preventing the oxidative damage especially under the ambient
toxic medium (Kodama et al., 1976). Amin et al. (2010) and Himri et al. (2011) also observed a significant increase in AST and ALT activities in Tartrazine treated rats. A significant increase in AST and ALT activities has also been observed in *C. punctatus* on exposure to mercuric chloride (Sastry and Sharma, 1980), *Tilapia mossambica* on exposure to arsenite (Rani et al., 2001), *C. lazera* on exposure to dyestuff and chemical wastewater (Abdel-Moneim et al., 2008) and *L. rohita* on exposure to anthracene (Vasanth et al., 2012). Enhancement of the activity of transaminases in the present fish may have been to provide the oxaloacetic acid, pyruvate, α-ketoglutarate and glutamic acid for the increased energy demand under the stress of the present dye as suggested by Begum (2004), Tilak et al. (2005) and Velmurugan et al. (2008). Decrease in the activity of ALT in the present fish may have been due to lipid peroxidation caused by accumulation of ROS under the stress of the dye as the decline was dose dependent. Many azo dyes have been reported to be cytotoxic (Ruparelia et al., 1999; Srivastava et al., 2004) therefore, the observed decrease in the activity of both AST and ALT in muscle during the short term exposure and the recovery period following it along with a decline in liver and gill during the recovery period after subchronic exposure may be due to damaged cells that are no longer capable of synthesizing both these enzymes as reported in the study of Abhijith et al. (2016). A decrease in the activity of aminotransferases in the liver of fish *Oreochromis mossambicus* due to liver damage on exposure to organophosphorus pesticide (RPR – II) has also been reported by Rao (2006).

GST is a well-known enzyme of phase II of the detoxification processes which conjugate glutathione to certain xenobiotic compounds or to their metabolites (Almar et al., 1998). Fish cells usually try to remove pollutants by means of GST (Ji et al., 2012) which could be the reason for the dose dependent increase in the activity of this enzyme in kidney, gill and brain of the present fish after 96h and in liver, kidney and gill by 150th day of subchronic exposure. Highest increase in GST activity at 10 mg/l dye (gill-39.15%) after 96h exposure was only 1.5 fold less than the highest increase during the subchronic exposure at 2.5 mg/l dye (kidney- 59.87%). This indicates that duration of exposure also had a marked effect on GST. Same trend of increase and decrease during the recovery periods after both the exposures shows that the fish tried but could not
recover from the stress of the dye even 90 days after exposure. Dose dependent increase in the activity of GST in kidney throughout the study could probably be due to the effort of the exposed fish to defend the body against the oxidative damage caused by the dye (Sun et al., 2006; Cazenave et al., 2009; Liu et al., 2009) and prolonged increase till the end of the recovery periods could be a metabolic adaptation for removal of breakdown products of AB-1 from the body. Simultaneous increase in GST and CAT in the liver and kidney of the fish during the subchronic exposure shows a shift towards detoxification mechanisms under the stress of the dye as suggested by Atli and Canli (2010). Raised GST activity has been associated with defensive adaptation of organisms to the presence of a variety of organic compounds in the environment by Vander Oost et al. (2003). Azo dye induced increase in the activity of GST in fish has been observed by Batzinger et al. (1978); Benson et al. (1979); Oh and Lee (1981); Yin et al. (2007) and Peng et al. (2010). GST induction has also been reported in fishes on exposure to organic contaminants or pesticides (Beyer et al., 1996; Grinwis et al., 2000; Schlezinger and Stegeman, 2000; Stephensen et al., 2000; Elia et al., 2002; Pena-Llopis et al., 2003; Ballesteros et al., 2009). Reversal in the trend of GST in liver, gill and brain during the subchronic exposure in comparison to 96h exposure may be due to lower doses of the dye during this period. The liver is involved in biotransformation of xenobiotics, therefore the stress might have led to depletion of GST (the main enzyme for detoxification) in it after 96h as the selected doses of AB-1 were lethal to the fish. The dose dependent increase in GST in liver, kidney and gill during the subchronic exposure and the recovery period hints towards involvement of this enzyme for detoxification of the present dye. Muscle showed a dose dependent decline throughout the study, although the dose for subchronic exposure was ¼ of the highest dose for 96h exposure but the highest decrease in GST in muscle was ~14 fold more. This clearly shows more impact of duration of exposure and was clearly evident from behavior of the dye exposed fish. Initially on exposure to the dye the fish swam vigorously and banged into the walls of the pool but then settled in a corner and moved very little indicating a stress on the nervous system and muscles of exposed fish. Decreased activity of GST has also been observed in the spleen of P. microps exposed to 3,4-DCA (Monteiro et al., 2006), in the liver, kidney and brain of goldfish exposed to Roundup (Lushchak et al., 2009),
in the liver of *Prochilodus lineatus* exposed to Roundup (Modesto and Martinez, 2010) and in the liver of zebrafish exposed to textile effluent (Wenjuan *et al.*, 2012). GST transfers GSH groups to proteins to target them for cellular export and subsequent metabolism and detoxification (Dringen, 2000; Dickinson and Forman, 2002; Hayes *et al.*, 2005). The observed decline in protein content for meeting the energy demand under the stress of AB-1 could also be responsible for a decline in GST in muscle throughout the study.

AChE is a serine hydrolase whose primary role is to hydrolyze and to modulate the amount of neurotransmitter acetylcholine in cholinergic synapses (O’ Brien, 1967). A dose dependent decline in the activity of AChE in all the tissues of the exposed fish could be due to inhibition of this enzyme by the present dye. The inhibition of AChE results in buildup of acetylcholine within the nerve synapses leading to a variety of neurotoxic effects and decreased cholinergic transmission (Milesen *et al.*, 1998). This can be dangerous since it will impact feeding capability, swimming activity, identification, avoidance of predators and spatial orientation of the species (Uner *et al.*, 2006). The present dye also strongly declined swimming movements as well as feeding intensity of the test fish during the exposure and the stress continued till 90 days post exposure. Malla Reddy *et al.* (1992) suggested that the inhibition of AChE activity with a concomitant increase in acetylcholine (Ach) content in the tissues is an implication of greater disruption to the activity of the central nervous system. Many workers have reported that dyes and their derivatives are complex inhibitors of AChE (Kucukkilinc and Ozer, 2007; Mansour *et al.*, 2010). Similar inhibition has also been observed, due to the stress of insecticides and heavy metals by other workers (Dutta *et al.*, 1995; Kumar and Chapman, 2001; Rao *et al.*, 2003; David *et al.*, 2009; Tilton *et al.*, 2011). Ballinger *et al.* (2005) suggested that oxidation of amino acids and lipids under the stress of pollutants led to inactivation of AChE. More decline in liver, kidney and gill during the present study could be due to a reduction in blood flow to these tissues under the stress of AB-1. Thomaz *et al.* (2009) reported that inhibition of AChE would likely result in continuous stimulation of neural-muscular junctions and could cause sphincters at the base of the efferent filament arteries to constrict and reduce blood flow through them. In the present study also no mortality was observed at 4 mg/l dye after 96h but 10-30%
mortality was observed during the subchronic exposure to 0.625-2.5 mg/l dye. Corbett (1974) suggested that damage to the central nervous system might cause uncontrolled hormonal release that could result in mortality of stressed animals possibly due to degeneration of many biochemical and physiological functions.

A significant increase over control in the activity of AcP in all the tissues and that of AKP activity in gill, muscle and brain throughout the present study could be due to dye induced cell damage as the increase was dose dependent. Actually when a cytotoxic agent like an azo dyes induces necrosis/apoptosis in the exposed animals it leads to an increase in the mobilization of lysosomes, which in turn leads to an increase in the AcP and AKP in the tissues (Rao, 2006). Decline in AcP activity of gill and that of AKP in the liver and kidney of the exposed fish could also be due to the stress of AB-1. Previous literature shows variation in the increase or decrease in AcP/AKP under stress, Sekar et al. (2008) observed an increase in AcP activity and a decline in AKP activity in the brain of S. hydrodroma on exposure to textile dye industry effluent, while Amin et al. (2010) reported a dose dependent increase in AKP activity in Tartrazine treated rats. Increased AcP as well as AKP activity in stressed fishes has been reported by other workers (El-Sayed and Saad, 2008; Vasanth et al., 2012). A decline in the activity of AKP in liver and kidney in the current study might be due to breakdown of glycogen to meet the energy demand under stress or due to a decrease in the rate of trans phosphorylation or uncoupling of oxidative phosphorylation (Saha and Kaviraj, 2009). Shaikila et al. (1993) have inferred that severe acidosis might have been responsible for inhibition of AKP in the liver of Sarotherodon mossambicus which in turn be an adaptation of the fish to meet the energy demand via anaerobic breakdown of glycogen. Disturbance of mitochondrial functions under the stress of present dye might have caused decline in AcP and AKP of the experimental fish as uncoupling of phosphorylation has been reported to result in a decline of these enzymes under the stress of pollutants by Parthasarathi and Karuppasamy (1998).

Glutathione exists in two states, GSH and GSSG, GSH is the first line of defense against oxidative stress, while GSSG, is an oxidized product of GSH. The increase in GSH in the liver and muscle of the present fish throughout the study could be an adaptive mechanism through an increase in its synthesis under the oxidative stress of
present dye (Doyotte et al., 1997; Zhang et al., 2004). The observed increase in GSH content can be attributed to the regeneration of GSH by GR as an adaptive and protective response to the dye induced oxidative stress as suggested by Wu et al. (2011). More decline of GSH in kidney and gill during the recovery period seems probably to be an indication of its exhaustion due to involvement in Phase II biotransformation and gets confirmation from the corresponding increase in GST activity in the present study. The depletion of GSH in kidney, gill and brain could be either due to high GSH utilization for conjugation and/or participation of GSH as an antioxidant in neutralizing free radicals (Yonar, 2011). Severe oxidative stress of AB-1 may have suppressed GSH levels due to the loss of adaptive mechanisms and the oxidation of GSH to its oxidized form, GSSG (Zhang et al., 2005). Azo dye induced decrease in the activity of GSH has been observed in hepatopancreas, haemolymph and ovary of S. serrata (Vijayavel et al., 2004), in liver of C. auratus (Yin et al., 2007), Brycon amazonicus (Avilez et al., 2008) and goldfish (Liu et al., 2009). Depletion of GSH might also be contributed by increased transportation of GSSG out of the cell because protective and adaptive roles of GSH against oxidative stress-induced toxicity are well established in aquatic animals (Otto and Moon, 1995). In the present study, level of GSSG also increased in all the tissues throughout the study. The increased level of GSSG could be explained by the increased activity of GR and GPx in the tissues of the fish on exposure to AB-1. Oxidation of GSH to GSSG is catalyzed by GPx and regeneration back to GSH by GR at the expense of NADPH, obtained from pentose phosphate pathway (Keppler et al., 1997). If regeneration of GSSG is higher than its reduction to GSH by GR then GSSG starts accumulating, and is translocated outside the cell by specific transporters to avoid NADPH exhaustion, which consequently leads to depletion of GSH (Kretzschmar, 1996). A dose dependent depletion in GSH in kidney and gill and prolongation of decline during the recovery period in the present study is probably due to the fact that the exposed tissues are unable to cope up with the high consumption of GSH in conjugation reactions (Tramboo et al., 2011) not only during the exposure but for quite a long time post exposure.

Malondialdehyde (MDA) is an essential marker for the determination of oxidative stress. It is produced from the lipid peroxidation of polyunsaturated fatty acids
and the degree of lipid peroxidation can be evaluated by the amount of malondialdehyde produced in the tissues. Reactive oxygen species degrade polyunsaturated lipids, resulting in the formation of malondialdehyde (Pryor and Stanley, 1975). Increase in MDA in all the tissues in a dose and duration dependent manner during the present study indicates induction of lipid peroxidation by AB-1 in the tissues of exposed fish. The increase in MDA can most likely be ascribed to an excessive production of ROS that could have resulted in leakage of antioxidant enzymes (Ibrahim, 2015). It has been reported that elevated ROS level in tissues leads to cellular damage when the rate of its generation surpasses the rate of its decomposition by antioxidant defense systems (Ayoola et al., 2012). The MDA content significantly increased, indicating that although enzymes such as SOD and CAT in the antioxidant defense system took part in scavenging the free radicals, plenty of free radicals remained, resulting in the accumulation of the lipid peroxides in the AB-1 exposed fish. Nair et al. (1986) and Wenjuan et al. (2012) also correlated increase in MDA to peroxidation (oxidative damage) of the lipid membranes in the exposed fish. Azo dye induced increase in the activity of MDA has been observed in rats, S. serrate and C. auratus by other workers also (Oh and Lee, 1981; Vijayavel et al., 2004; Shi et al., 2005; Peng et al., 2010). Vijayavel et al. (2004) reported that in naphthalene exposed crab S. serrate, an increased level of LPO was due to the microsomal metabolism of aromatic hydrocarbons and microsome mediated redox cycling which gave rise to oxyradicals capable of oxidizing membrane lipids. Most components of cellular structure and function are likely to be potential targets of oxidative damage and the most susceptible substrates for autoxidation are polyunsaturated fatty acids of the cell membrane, which undergo lipid peroxidation. The reactive aldehydes produced during lipid peroxidation can diffuse from production site and cause damage to inter and intra cellular targets (Esterbauer et al., 1990). An increased MDA level has also been observed in rats exposed to cadmium and naphthalene (Bagchi et al., 2002). Increase in lipid peroxidation seems to be due to an inhibitory effect of AB-1 on mitochondrial electron transport system leading to stimulation of the production of intracellular reactive oxygen species as suggested by Stohs et al. (2000). In the present study, higher increase in MDA in all the tissues after 150 days of subchronic exposure and the
recovery period following it gives a clear indication about the duration dependent accentuation of the stress of even minute doses of AB-1. El-Tohamy (2012) suggested that extent of oxidative damage depends not only on the nature and amount of ROS but also depends on the moment and duration of ROS exposure along with ROS scavengers.

5.2 Water and lipid soluble antioxidants

In biological systems the oxidation processes are balanced by the presence of natural antioxidants. Two types of natural non-enzymatic antioxidants (water soluble and lipid soluble) scavenge free radicals in the organisms. These antioxidants reduce free radicals and get oxidized themselves, then these may be reduced to their active forms by other reducing systems. Water soluble antioxidants include ascorbic acid (vitamin C), glutathione, lipoic acid and uric acid while lipid soluble antioxidants include carotene, α-tocopherol (vitamin E) and ubiquinol (coenzyme Q). Lipid soluble antioxidants are supposed to act as highly efficient scavengers, against lipid peroxyl radicals, which are formed within the lipoprotein as a consequence of free radical chain reaction of lipid peroxidation (Kumar, 2011).

A decrease in the water soluble antioxidants of blood and muscle and an increase in lipid soluble antioxidants in all the three tissues after 96h and in liver and blood during subchronic exposure could be due to the increased production of ROS under the stress of present dye. Buettner and Jurkiewicz (1996) suggested that lipid soluble antioxidants donate a hydrogen atom to a fatty acid based free radical more readily than does an unoxidized fatty acid. Water soluble antioxidants interacting between the aqueous and lipid phases can then reduce the lipid soluble antioxidants so that they can continue to participate in antioxidative reactions. The ability of an antioxidant to reduce another antioxidant or a lipid derived radical is determined by its reduction potentials and is considered to be an indicator of stress. Upadhyay and Panda (2010) suggested that ROS utilized large amount of GSH, depressed re-generation of GSH or caused leakage of GSH from the tissue because ROS and free radicals interacted with antioxidants during stress conditions to terminate the chain reactions. Decrease in water soluble antioxidants (Parvez and Raisuddin, 2006; Ali, 2012; Ekambaram et al., 2012; Sripriya et al., 2014) and an increase in lipid soluble
antioxidants (Passi et al., 2002; McLean et al., 2005) has been observed in stressed animals by other workers also. Maximum decline in water soluble antioxidants and maximum increase in lipid soluble antioxidants was observed in blood of the fish during the present study. Water-soluble antioxidants cannot enter the lipid moiety of low density lipoprotein (LDL) and are thus less efficient as these are principally unable to encounter most of the lyophilic radicals; however, such a compound may act in a synergistic manner with lipophilic antioxidants by regenerating them. The observed increase in MDA in all the tissues of AB-1 exposed fish suggests that inadequate detoxification mechanisms may have caused overproduction of reactive radicals in the fish that induced oxidative damage in its tissues. Increase in lipid soluble antioxidants in all the tissues of the fish also indicates towards the efforts of the fish to overcome the stress of exposure to the present dye. Lipid soluble antioxidants, α-tocopherol and tocotrienols the isomers of vitamin E, act synergistically with many other antioxidants. These are powerful chain-breaking antioxidants that inhibit ROS induced generation of lipid peroxyl radicals, for protecting cells from peroxidation of PUFA in membrane phospholipids and from oxidative damage of cellular proteins (Shrivastava, 2012). Lipid radicals are converted into hydroperoxides by α-tocopherol, and α-tocopherol itself changes into α-tocopheryl semiquinone radical. The α-tocopheryl semiquinone radical can react further with another lipid radical to produce more hydroperoxide and methyltocopherylquinone or react with another α-tocopheryl semiquinone radical and produce α-tocopherol dimer. The methyltocopherylquinone is unstable and will yield α-tocopherylquinone, however, the α-tocopheryl dimer continues to produce antioxidant activity (Reische et al., 1999) for a long time to protect the cells under stress. Coenzyme Q, another lipid-soluble antioxidant, is an important part in the electron transport chain. It is able to sustain efficiently the chain-breaking antioxidant capacity of Vitamin E by regenerating it from tocopheroxyl radical (Winston and Di Giulio, 1991). This could also be the reason for an increase in lipid soluble antioxidants in all the tissues after 96h exposure to the dye. On the other hand, decrease in both water and lipid soluble antioxidants in muscle during the subchronic exposure and the recovery period could be responsible for no movement and reduced feed intake by the AB-1 exposed fish. Increase in water soluble antioxidants in liver during the exposures as well as the
recovery periods also seems an effort of the fish for overcoming the stress of the dye. Liver is the main site of detoxification and water soluble antioxidants have been reported to act primarily in cellular fluid in particular for combating reactive oxygen species to protect biomembranes from peroxidative damage induced by pollution. They are also involved in regeneration of other small molecule antioxidants, such as α-tocopherol, glutathione, and β-carotene (Halliwell, 1996). This could probably be the reason for the observed increase in water soluble antioxidants in the liver during the recovery periods of the present study.

5.3 Proteins, free amino acids and secondary structure of proteins

Proteins, the basic building blocks of all animals, are one of the most complex nitrogen containing macromolecules (Jauncey, 1982). These biomolecules are the major source of energy during chronic stress conditions (Umminger, 1970) and play a vital role in tissue binding and repair (Senthilkumar et al., 2007). Proteins vary in accordance with the number and sequence of amino acids, which are the basic units of a protein molecule (Von Wachtendonk and Kappler, 1977). Free amino acids (FAA) that are produced as a result of proteolysis of the dietary proteins are used by animals to synthesize their own proteins. But there is variation in the levels of free amino acids under different physiological conditions hence increase or decrease in the content of free amino acids provides valuable information at the tissue level in stressed animals (Magar and Shaikh, 2012). Protein degradation and subsequent utilization of the released amino acids for anaplerotic reactions or energy production is responsible for changes in the concentration of total free amino acids (Bais and Lokhande, 2012).

The present dye drastically affected concentration of proteins in various tissues of the fish. The decline in protein clearly hints towards an effect of this dye on the activity of most of the enzymes and other functions of the cells. Because proteins are the constituents of the cell membrane, have a major role in the interactions between intra and extracellular media and, also participate in the intricately balanced sub cellular activities (Soundararajan and Veeraiyan, 2010). Proteins are one of the main energy sources in fishes and play an important role in the maintenance of blood glucose. In the present study, a continuous decrease in protein content during the recovery periods after
both the exposures may be due to breakdown of proteins in generating energy for the essential processes of the dye exposed fish. Higher decline in protein content during the subchronic exposure and both the recovery periods could also be related to a decline in feed intake by the exposed fish. It is known that under the stress conditions, even the proteins that are consumed by the fish are not stored in the body tissues but are mobilized along with body proteins to produce glucose by the process of gluconeogenesis to meet the extra demand for energy (Tripathi et al., 2003; Virk and Sharma, 2003; Naveed et al., 2010). The lowering of proteins and elevation of FAA are apparently inter-related and are indicative of metabolic utilization driving a possible source of energy to meet the energy demand under stress (Prashanth and Neelagund, 2007). Decreased protein content has also been observed in C. carpio, O. striatus, C. mrigala and C. punctatus on exposure to sodium cyanide, cadmium chloride, free cyanide and mercuric chloride, respectively (Prashanth and Neelagund, 2007; Jayanthi and Selvakumar, 2011; Bais and Lokhande, 2012; David and Kartheek, 2014). Maximum decline in protein in muscle after both the exposures, and 1.5-2 fold higher decline during the recovery periods of the present study seems to be responsible for weakening and lethargy of the AB-1 exposed fish. Low content of proteins in muscles clearly reflects difference in the rate of synthesis and degradation of protein (Arai, 1974) that intturn lowers working capacity in stressed animals. Thenmozhi et al. (2011) reported that gradual decrease in the protein content as observed in the present fish till the end of the recovery period suggested disruption of carbohydrate metabolism, destruction of protein and protein synthesis machinery and inhibition of ATP synthesis. Toxicant poisoning has been related to reduction in protein by Baskaran (1980); Jana and Bandyopadhyaya (1987) and Vincent and Ambrose (1994).

The steady-state concentration of amino acids in the tissues depends on the rates of their degradation and production (Lim et al., 2001). In the present study, increase in amino acids in all the tissues of the dye exposed fish corresponded to the decrease in protein level. The increased free amino acid level clearly indicated breakdown of protein for energy requirement and for incorporation of amino acids in protein synthesis under the stress of present dye. Increase in the free amino acid level due to stress has been expressed as a consequence of the higher catabolic activity of protein to meet the
Discussion

high energy demand by breaking down the protein into free amino acids by Parthipan and Muniyan (2014). Similar results were observed in *O. striatus*, *C. punctatus*, *L. rohita* and *C. mrigala* on exposure to cadmium chloride, mercuric chloride, aluminium chloride and zinc cyanide (Jayanthi and Selvakumar, 2011; Bais and Lokhande, 2012; Shwetha et al., 2012; Selvam et al., 2014). Decrease in the concentration of some amino acids during the subchronic exposure (aspartic acid, glutamic acid, lysine, histidine, alanine, leucine and glycine) and during the recovery period after 96h exposure (valine and methionine) as well as subchronic exposure (arginine, isoleucine and tryptophan) in the present study indicates the simultaneous decrease in the rates of proteolysis and amino acid catabolism (Lim et al., 2001).

The sulfur containing amino acids get partially oxidized during acid hydrolysis (Van de Poll et al., 2005) and this might be the reason for the lower content or disappearance of cysteine and methionine in the tissues of present fish under the stress of AB-1. The disappearance of asparagine and glutamine from the amino acid profile in the current study may have been due to their conversion to parent dicarboxylic acids during acid hydrolysis and thus, the peak detected for glutamate and aspartate may be considered as the composite values for their amides (James and Kumar, 2013). The elevated levels of glutamine during the subchronic exposure may have been for its utilization as a precursor for the synthesis of essential proteins, or towards gluconeogenesis, glycogenesis and keto acid synthesis (Murray et al., 1995). Glutamine is the major amino acid found in the circulatory system for carrying ammonia to and from various tissues but principally from peripheral tissues to the kidney, where the amide nitrogen is hydrolyzed by the enzyme glutaminase, this process regenerates glutamate and free ammonium ion, which is excreted in the urine (Campbell, 1997). In the present study, increased glutamine content due to AB-1 exposure might be due to triggering of the operation of detoxification of ammonia, chiefly by way of formation of less toxic nitrogenous substances, namely urea and glutamine (Krebs, 1980). Transaminases are considered as an index of gluconeogenesis, stimulation of the activity of both AST and ALT under the stress of AB-1 in the present study suggests increased mobilization of free amino acids into gluconeogenesis and glutamate formation and their feeding to TCA cycle (Prashanth and Neelagund, 2007).
FTIR is one of the important techniques for the analysis of secondary structure of polypeptides and proteins. The most widely used modes in protein structure studies are amide I, amide II and amide III, which are located between 1700-1600 cm\(^{-1}\), 1600-1500 cm\(^{-1}\) and 1500-1000 cm\(^{-1}\), respectively (Warnau et al., 1996). The amide I band arises principally from the C=O stretching vibration of the peptide group and the amide II band is primarily N-H bending with a contribution from C-N stretching vibrations. The amide III absorption is normally weak and arises primarily from N-H bending and C-N stretching vibrations (Venkataramana et al., 2010). In the present study, the original spectra had complex multicomponent bands which overlapped into a broad unresolved absorption, and the individual component absorptions from condensed phase spectra could not be resolved by increasing spectral resolution, causing difficulty in band differentiation and their assignment. Therefore, the second-derivative spectra processes were performed to distinguish overlapping peaks and to achieve more precise calculation on band position and its area. The advantage of this process lies in that the data is de-noised to a great extent and overlapping is largely minimized as suggested by D’Souza et al. (2008). The changes in peak position of amide bands may quantitatively reflect alterations in the composition of protein structure (Susi and Byler, 1983). These vibrations are influenced by structure of the protein, which involves protein folding with hydrogen bonding between peptide bonds. Rice-Evans et al. (1991) suggested that all the constituent amino acid side chains in proteins are susceptible to free radicals, but some are more vulnerable than others. Thus, exposure of proteins to free radical-generating systems may induce secondary structural changes. The spectrum of control and AB-1 treated samples were observed to differ in the shape of absorbance peaks, indicating obvious changes in structure and contents of proteins due to the stress of this azo dye. Azo dyes have been demonstrated to produce ROS in the exposed animals (Himri et al., 2011) which could be the reason for the observed changes in the area and width of amide I, II and III peaks in the present study.

Amide I and II bands were affected more than the Amide III bands by the present dye. Higher doses of this dye (during 96h exposure) caused more effect on liver (Amide II) and blood (Amide I) whereas during the subchronic exposure and the recovery periods after both the exposures more effect was observed in muscle. The shift
observed for the amide bands in the tissues of exposed fish indicates that the present dye induced important structural alteration in the existing proteins as suggested by Toyran et al. (2008). Significant decline in the area of amide I band (1654 cm\(^{-1}\)) indicates a dye induced disturbance in the α-helical structure of the proteins of the present fish (Palaniappan and Vijayasundaram, 2008). Further, decline in the area of amide II band observed at 1543 cm\(^{-1}\) reflects the loss of protein level due to oxidation in the tissues of the dye exposed fish (Takahashi et al., 1991; Cakmak et al., 2006; Akkas et al., 2007). The decrease in the band areas of amide I and II has been observed in the liver of \(L.\) rohita on exposure to arsenic (Palaniappan and Vijayasundaram, 2008), in gill of \(L.\) rohita on exposure to zinc (Palaniappan et al., 2010) and in muscle of \(L.\) calcarifer on exposure to nickel and mercury (Senthamilselvan et al., 2012). Toyran et al. (2005) suggested that loss of function of protein may result from a change in critical side chains or from a break in the hydrogen or disulfide bonds, which maintain the secondary and tertiary structures. This break can lead to a partial unfolding of the tightly coiled peptide chains that result in a disorganization of the internal structure. This is reflected as shift in peaks or change in peak area.

The decline in the band area and frequency of 1450 cm\(^{-1}\) in the present study could be due to the disordering of the CH\(_2\) bending vibrations of proteins due to the toxic effect of the dye. However, a decline in the area but an increase in the frequency of 1385 cm\(^{-1}\) band might be due to salt bridge disruption, because it is well established that this vibrational mode shifts to higher frequencies as the interactions of COO\(^{-}\) groups with their oppositely charged partners weaken under stress (Carmona et al., 2008).

Proteins play a vital role in the physiology of living organisms. All the functions of an organism are regulated by enzymes (proteins) and hormones (many are proteinaceous). If any alteration takes place in the protein turnover, it may have an adverse effect on the important and complex groups of biomolecules and food intake. This will further affect biological events that maintain homeostasis of the cell and body of an organism. Therefore, the content and composition of proteins of a cell is considered a diagnostic tool to determine the physiological phases of a cell (Manoj and Ragothaman, 1999).
Alterations in the antioxidative and detoxification enzymes along with changes in secondary structure of proteins in the AB-1 exposed fish prolonged till 90 days post exposure in the present study. It seems that the present dye may have impaired organelle and cell structure and caused denaturation of proteins which put the fish under further stress for a long time. It is clear that such effects of the dye will put the fish under further stress with a slight change in their environment. Therefore, there is a strong need to regulate discharge of such dyes in natural waters. The data clearly show that SOD, water soluble antioxidants along with valine and amide I band in the liver and muscle can act as biomarkers for the stress of AB-1. Liver was observed to be the most sensitive during the short term exposure (96h) while muscle was most sensitive during the subchronic exposure (150 days) to AB-1 as well as during the post exposure period of 90 days.