6. DISCUSSION

Spectral analysis

The principal method for spectral analysis of CYP 450 involves what is termed the ferrous-CO difference spectrum. In our study, two soret peaks were seen in the dithionite reduced spectra of liver microsomes in control and experimental groups of all 3 fish studied, *C. punctatus*, *H. fossilis* and *C. batrachus*. These peaks consisted of the characteristic absorbance at around 450 nm for the reduced CYP 450-CO complex and the other of lesser magnitude at around 420-425 nm (Fig. 5.1, 5.2 and 5.3 a and b). The maximum absorbance was noticed at 1–2 min and then a gradual decrease in absorbance was seen with the increase in time interval. This time interval corresponds to the duration of incubation necessary for maximum reduction of the enzyme by sodium dithionite.

The CYP 450 is converted to a stable denatured state CYP 420 under long periods of incubation thus adding to the absorbance at 420-425 nm or may be possibly due to the absorbance of contaminating haemoglobin or cytochrome b_5 (Klingenberg, 1958; Garfinkle, 1958; Omura and Sato, 1962; Arinc and Cakir, 1999). Not only CYP 450 binds CO in its reduced (ferrous) form, haemoglobin and other hemoproteins also bind CO in reduced form (Schenkman and Jansson, 2006; Guengerich *et al*., 2009).

The spectral absorbance at 420-425 nm was also described in a study with Neotropical freshwater fish species and adopted tissue perfusion method to decrease the influence of haemoglobin contamination of microsomes (Leitao *et al*., 2000). Similar unusual spectra were obtained when fish, *Hypostomus punctatus* and *Ancistrus multispinis* were examined for spectral analysis (Klemz *et al*., 2010). The peak at 420-425 nm in the current study may be due to the haemoglobin contamination since tissues could not be perfused adequately. Overall, the spectral analysis with the peak absorbance at around 450 nm also indicated the presence of active CYP 450 enzyme in hepatic microsomes.

Multiple forms of cytochrome P450

The spectral analysis of *H. fossilis* treated with naphthalene, phenobarbitone, deflazacort and acetone revealed dissimilar absorption peaks in comparison to that of control fish. The control fish showed a maximum peak at 450 nm, naphthalene at 451 nm, phenobarbitone at 452 nm, acetone at 450 nm and that of deflazacort treated at 449 nm respectively (Fig. 5.4

and Table 5.1). The hepatic CYP 450 system consists of a family of inducible isoenzymes that possess different spectral and immunological characteristics, substrate preferences and primary protein structures (Wickramasinghe *et al.*, 1980; Guengerich *et al.*, 1982). Purification and separation procedures for CYP 450 often take advantage of the fact that different prominent forms are induced by particular xenobiotics and one to five forms has been accomplished in rat liver microsomes treated with polychlorinated biphenyls, phenobarbital, 3-methylcholanthrene and β-naphthoflavone, which displayed a spectral characteristics of CYP 450 peaks ranging from 446 to 452 nm (Kotake and Funae, 1980; Lau and Strobel, 1982; Seidel and Shires, 1986).

In the present study, *H. fossilis* treated with naphthalene (66.274±23.059 pmole resorufin formed/mg protein/minute) displayed a significant induction ($p<0.05$) in EROD activity when compared with the control (17.989±12.034 pmole resorufin formed/mg protein/minute) and phenobarbitone, acetone and deflazacort showed marginal increase in EROD activity (Table 5.2). So the fish, *H. fossilis* treated with naphthalene indicated its specificity to induce CYP1A isoform. Pacheco and Santos (2002) have reported that naphthalene also acts as a specific inducer of CYP1A in European eel. Although several isoforms have been identified in fish, CYP1A has received the most attention as the major hydrocarbon-inducible CYP 450.

CYP1A induction is mediated by the Ah receptor (AhR), a xenobiotic-binding protein present in the cytosol (Lewis, 2001). Well-established inducers of CYP1A and EROD are organic contaminants belonging to polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), nitrated polyaromatic hydrocarbons (NPAHs), dioxins, some pesticides (Machala *et al.*, 1997; Jung *et al.*, 2001) and have been so far proved to be the most sensitive indicator (Schlenk and Di Giulio, 2002).

There were no significant difference in N, N-dimethylaniline demethylase (N,N-DMA) activity between the control (0.054±0.045 nmole formaldehyde formed/mg protein/minute) and the phenobarbitone treated group (0.060±0.013 nmole formaldehyde formed/mg protein/minute) of fish, *H. fossilis* as well as the fish treated with naphthalene, acetone and deflazacort. The acetone treated group (0.036±0.005 nmole formaldehyde formed/mg protein/minute) showed a slightly lower activity but was not significant (Table 5.2). Thus the inducers phenobarbitone, naphthalene, acetone and deflazacort have no effect on CYP2B isoform responsible for mediating N,N-DMA activity. Goksoyr and Forlin (1992) concluded that subfamily CYP2B is totally absent in fish, however, over the last decades, fish liver microsomes have been shown to metabolize prototypical mammalian CYP2B substrates

(Elskus and Stegemann, 1989; Haasch *et al.*, 1994). In mammals, phenobarbital is an *in vivo* inducer of the CYP2B family, whereas in teleosts CYP 450 induction by phenobarbital is unclear (Sadars *et al.*, 1996). In contrast, Stegemann *et al*. (1997) reported the presence of CYP2B like proteins in four tropical fish species (*Perca fluviatilis*, *Cephalopholis cruentata*, *Haemulon aurolineatum* and *Abudefdud saxatilis*) using immunoblot assay with rat CYP2B1 and CYP2B-like scup P450B antibodies.

Acetone treatment in fish, *H. fossilis* showed significant induction ($p<0.01$) in aniline hydroxylase (AH) activity (0.277±0.008 nmole p-aminophenol formed/mg protein/minute) as compared to control (0.037±0.028 nmole p-aminophenol formed/mg protein/minute). The fish treated with naphthalene and phenobarbitone showed a marginal decrease in aniline hydroxylase activity while deflazacort showed a marginal increase in the activity (Table 5.2). CYP2E1 metabolizes a number of low-molecular-weight pharmaceutical and other xenobiotic compounds, like acetaminophen, ethanol, isoniazid, halogenated anaesthetics, acetone and benzene (Tu *et al*., 1983; Lee *et al*., 1996; Gonzalez, 2005). The present study showed that in *H. fossilis* acetone induces AH activity indicating the presence of CYP2E1. CYP2E1 is also reported to be induced by starvation in animal models (Lieber, 1997). CYP 450 dependent aniline hydroxylation enzyme system is known to be a possible way for metabolism in the liver of fish species and aniline hydroxylase is known to be a member of mixed-function oxidases catalyzing the transformation of toxic aniline to nontoxic *p*-aminophenol (Zen and Korkmaz, 2009).

Deflazacort (synthetic corticosteroid) treated *H. fossilis* showed significant ($p<0.01$) induction of erythromycin N-demethylase (ERND) activity (3.129±0.545 nmole formaldehyde formed/mg protein/minute) whereas the naphthalene, phenobarbitone and acetone treated groups showed lower activity than that of the control (0.898±0.062 nmole formaldehyde formed/mg protein/minute) with significant difference seen in phenobarbitone (0.506±0.103 nmole formaldehyde formed/mg protein/minute; $p<0.05$) and acetone (0.127±0.22nmole formaldehyde formed/mg protein/minute; $p<0.01$) treated group (Table 5.2). CYP3A plays an important role in the metabolism of endogenous substances and xenobiotics including pharmaceuticals and is inducible by steroidal chemicals and a variety of naturally occurring compounds and synthetic glucocorticoids and macrolide antibiotics (Wickramasinghe, 1990; Quattrochi and Guzelian, 2001). Induction of CYP3A expression by dexamethasone has been shown to occur in fish (Tseng *et al*., 2005). As there is increase in ERND activity after treatment of the fish with deflazacort, a synthetic corticosteroid, it can be

concluded that CYP3A4 is present and induced in *H. fossilis*. Strong similarities in the structure and catalytic function of CYP3A4 between fish and mammals have been reported through the use of polyclonal antibodies (Popovic *et al*., 2013).

The inducers, naphthalene, phenobarbitone, acetone and deflazacort administered to fish, *H. fossilis* did not have a profound effect on other CYP 450 isoform apart from its chosen specific isoform. The CYP 450 family- CYP1A, CYP2E1 and CYP3A4 show similar biotransformation reaction as that reported for various mammalian species (Chan *et al*., 2009) but CYP2B did not follow the trend though still a lot of studies have to be conducted.

Studies with various mammalian species have clearly confirmed the presence of multiple forms of hepatic (Lu and Levin, 1974) and extrahepatic (Wolf *et al*., 1978) CYP 450 and have shown that various CYP 450 isoforms have divergent substrate specificities and are under different regulatory control. However, almost every isoform has its specific substrate that can be used for its identification (Anzenbacherova and Anzenbacher, 2001; Lewis, 2001). In contrast, bacterial and mitochondrial CYP 450 isoforms are highly substrate-specific (Lewis 2001). Baron *et al*. (2001) reported the presence of multiple CYP 450 isoforms in humans, especially CYP1A1, 1B1, 2B6, 2E1, and 3A by carefully studying on RNA, protein, morphologic and catalytic level by reverse transcriptase-PCR, immunoblot, immunohistochemistry and catalytic assays.

Induction of microsomal mixed-function oxidase system can influence the metabolism and toxicity of xenobiotics in several ways. Due to the similarities between xenobiotic metabolism in fish and mammalian species, it is not surprising that similar toxicological responses are observed with some chemicals in fish and mammals (Lech and Bend, 1980). However, some standard compounds that act in mammals as CYP 450 inducers are not acting as inducers in fish, such as phenobarbital or methoxychlor do not induce CYP2B or CYP3A, respectively (Ruus *et al*., 2002; Stuchal *et al*., 2006).

β-naphthoflavone (β-NF) is reported as a specific inducer of CYP1A in all of the vertebrate taxa (Prettia *et al*., 2001). All the 3 fish, *C. punctatus* (194.811±7.961 pmole resorufin formed/mg protein/minute), *H. fossilis* (255.415±9.651 pmol resorufin formed/mg protein/minute) and *C. batrachus* (185.252±22.159 pmole resorufin formed/mg protein/minute) treated with β-naphthoflavone displayed a significant increase ($p<0.001$) in EROD activity suggesting its specificity to induce CYP1A isoform (Table 5.4). Significant differences were also observed between control and all the 3 β-naphthoflavone treated fish

species in CYP 450 content. Prettia *et al*. (2001) have reported an increase in CYP 450 content and EROD activity in gilthead seabream after treatment with β-NF.

The fish, *C. punctatus*, *H. fossilis* and *C. batrachus* showed a negligible response towards β-naphthoflavone treatment in N, N-dimethylaniline demethylase and aniline hydroxylase activity compared to their respective control group indicating no effect on CYP2B and CYP2E1 isoform (Table 5.4). However, all the fish, *C. punctatus*, *H. fossilis* and *C. batrachus* administered with β-NF displayed an elevated level in erythromycin N-demethylase activity catalysed by CYP3A4 isoform though only *H. fossilis* (1.026±0.078 nmole formaldehyde formed/mg protein/minute) showed a significant difference ($p<0.05$) when compared to their respective control (Table 5.4). Husoy *et al*. (1994) have showed the occurrence of CYP3A-like isozymes in many organs of the body in control and β–NF treated cod by immunohistochemistry analysis.

All the 3 fish species, *C. Punctatus*, *H. fossilis* and *C. batrachus* administered with β-NF displayed an induction in CYP1A activity but had no major influence in CYP2B, CYP2E1 and CYP3A4 activities. The results from the current study suggest that fish CYP 450 responds differentially to different xenobiotics and indicated the presence of multiple forms of CYP 450 in fish (CYP1A, CYP2B, CYP2E1 and CYP3A4).

Acute toxicity (LC_{50})

The LC_{50} value represents the mortality of 50% of the tested organisms exposed to particular toxicants. Determination of LC_{50} value for the pesticide to a particular fish is important as they provide information about the safe level of pesticide use in the agricultural or household or any other application. In the present study, we also calculated the LC_{50} value of the pesticides cypermethrin (Ripcord 10% EC, pyrethroid), ethion (ethion 50 % EC, organophosphate) and dicofol (COLONEL S 18.5 % EC, organochlorine) in *C. punctatus*, *H. fossilis* and *C. batrachus*. The LC_{50} values of cypermethrin in *C. punctatus*, *H. fossilis* and *C. batrachus* was calculated to be 19.9 µg/L, 3.7 µg/L and 5.6 µg/L respectively (Table 5.5). However, different authors have reported different LC_{50} values in their study. Saha and Kaviraj (2003), Kumar *et al*. (2007) and Begum (2007) reported the LC_{50} value of cypermethrin as 0.67 μg/L, 0.4 mg/L, and 0.07 mg/L in *H. fossilis*, *C. punctatus* and *C. batrachus* respectively.

The LC_{50} values of ethion in *C. punctatus*, *H. fossilis* and *C. batrachus* was calculated to be 43.9 µg/L, 54.8 µg/L and 48.7 µg/L respectively (Table 5.6). However, Bhatnagar *et al*. (2003) reported the LC_{50} value of 0.43 mg/L for common carp, *Cyprinus carpio* when exposed to pesticide Match (Ethion-50% EC) which is higher than the value found in our study. The LC_{50} value of chlorpyrifos, the other class of organophosphate pesticide in fish *C. punctatus*, *C. batrachus* and *H. fossilis* was cited as 0.81 mg/L (Ali *et al*., 2008), 16.5 mg/L (Narra *et al*., 2011) and 2.84 mg/L (Khatun and Mahanta, 2014) respectively which is much higher than ethion reported in our study. The LC_{50} value of ethion in *C. punctatus*, *H. fossilis* and *C. batrachus* was lacking in literature.

The LC_{50} values of dicofol in *C. punctatus*, *H. fossilis* and *C. batrachus* was calculated to be 45.8 µg/L, 36.1 µg/L and 51.8 µg/L respectively (Table 5.7). The LC_{50} value, however, is lower than the 0.72 mg/L reported by Veeraiah *et al*. (2013) for *C. punctatus* exposed to dicofol 18.5% EC. The LC_{50} value of dicofol for *H. fossilis* and *C. batrachus* was lacking in literature though the LC_{50} value of endosulfan, another class of organochlorine pesticide was reported to be 0.75 mg/L (Singh and Srivastava, 1981), 0.6 mg/L (Tripathi and Verma, 2004) and 22.15 µg/L (Haloi *et al*., 2014) for *H. fossilis*, *C. batrachus* and *C. punctatus* respectively.

Of the 3 pesticides tested (cypermethrin, ethion and dicofol), cypermethrin was found to be the most sensitive to all the fish studied. It is in accordance with the previous study reported by Bradbury and Coates (1989), that fish have poor ability to metabolize and eliminate pyrethroid pesticide as compared to those of higher vertebrates. It is difficult to compare the toxicity of an individual pesticide to different species of fish as it is found to be influenced by several factors like physicochemical parameters of water (temperature, hardness, pH and dissolved oxygen content, etc), age, size and formulations of chemicals (Pandey *et al*., 2011).

Higher LC_{50} values are generally considered to be less toxic because greater concentrations are required to produce 50% mortality in animals (Hedayati *et al*., 2010). The acute toxicity test is used to determine the susceptibility and survival potential of animals to a toxic agent that produces a deleterious effect on a group of experimental organisms during a short-term exposure under controlled conditions, and to measure the effect of toxic chemicals in the biology of the tested species (Rani *et al*., 2011). Though acute toxicity test is not an accurate method of getting the exact value of LC_{50}, however, it is somewhat the best method till now to get an idea of the threshold level of pesticide toxicity to a particular fish or any aquatic organism (Haloi *et al*., 2014).

All the experimental fish, *C. punctatus*, *H. fossilis* and *C. batrachus* were observed to visit the surface of water much more frequently to gulf the air after the application of tested pesticide. Further dose-dependent abnormal behavioural changes in fish were noticed indicating the stages of stress response. The present study showed that the pesticide, cypermethrin, ethion and dicofol are highly toxic to fish, *C. punctatus*, *H. fossilis* and *C. batrachus* even if exposed to a minute concentration.

Liver somatic index (LSI), Microsomal protein content and CYP 450 content

The liver contains the bulk of the detoxification potential and it can be inferred that the addition of liver mass by the animals could be an effective metabolic investment directed towards increased overall functional capacity of the organ to allow for more efficient detoxification. In this study, all the experimental fish reflected an increase in their LSI values compared to that of the control group (Table 5.3, 5.8, 5.15 and 5.22). The LSI values were higher in all the 3 β-naphthoflavone treated fish, *C. punctatus*, *H. fossilis* and *C. batrachus* but the significant difference ($p<0.05$) was revealed only by *C. batrachus* (1.219±0.076) compared to their respective control group (Table 5.3).

All the cypermethrin treated fish displayed elevated LSI values but the significant difference ($p<0.05$) was shown by only 15 days treated group of *C. punctatus* (1.042±0.109) with 1.1 fold increase and *H. fossilis* (1.245±0.144) with 1.2 fold increase while *C. batrachus* showed no significant increase in any treated groups compared to their respective control group (Table 5.8). In case of ethion treatment, the significant difference ($p<0.01$) in LSI values were shown by 10 days (1.075±0.085) and 15 days (1.155±0.122) treated groups of *C. punctatus*, while *H. fossilis* and *C. batrachus* showed no significant increase in any of the treated groups (5, 10 and 15 days) compared to their respective control group. Highest elevation with 1.2 fold increase in LSI value was displayed by 15 days treated *C. punctatus* (Table 5.15). All of the dicofol treated fish displayed significant difference in LSI values when compared with their respective control group. Highest elevation with 1.5 fold increase was shown by 5 days (1.531±0.488) treated *C. batrachus* (Table 5.22).

The increase in LSI is commonly seen in fish exposed for longer periods of time to contaminants in both the laboratory and field (Whatley *et al.*, 2010). This increase is not unexpected since the liver is regarded as the main detoxification organ and functions in storing and metabolising toxicants. LSI has frequently been used as a biomarker for

examining fish exposed to environmental contaminants and also to determine the physiological status that can establish the welfare and condition of fish (Goede and Barton, 1990; Sole *et al*., 2009). Although, identification of the cause for LSI changes was not within the scope of this study, the higher LSI value in experimental fish compared to the control fish suggests that it may be due to the effect of pesticides. LSI values are generally elevated in vertebrates experiencing stimulation of hepatic microsomal CYP 450 for the detoxification of organic compounds and the elevation in LSI may be due to the altered allowance of energy reserves for detoxification of organic compounds (Miller *et al*., 2009).

The increase in LSI may be associated with the increased capacity to metabolize xenobiotics and may be considered as adaption to the presence of pollution rather than a dysfunction (Heath, 1995). This increase could also be linked to an increase in cell numbers, which is the response of the organism to the toxins leading to an increase in liver size coupled with pollutant exposure (Goede and Barton, 1990; Face *et al*., 2005). However, a histological assessment was not carried out on the liver, therefore, this increase in liver size could either be linked to an increase in the number (hyperplasia) or an increase in the size (hypertrophy) of the hepatocytes. Further investigation is needed.

The amount of microsomal protein per gram of liver is a critical scaling factor used in toxicology study models to extrapolate *in vitro* rates of metabolism to xenobiotic clearance *in vivo*. The liver is the main site of metabolic clearance and an accumulation of liver protein could result from an increased rate of synthesis or a decreased rate of breakdown. In this study, we found that the microsomal protein content was significantly higher in all the experimental groups of fish, *C. punctatus*, *H. fossilis* and *C. batrachus* when compared to their respective control group. The microsomal protein content increased with increasing days of exposure (5, 10 and 15 days) to the pesticide tested (Table 5.9, 5.16 and 5.23).

In cypermethrin exposed group, the highest elevation in microsomal protein content was shown by *C. batrachus* (4.994±0.385 mg/gm liver; $p<0.001$) with 1.6 fold increase followed by *H. fossilis* (4.981±0.633 mg/gm liver; $p<0.001$) with 1.5 fold increase and *C. punctatus* (4.125±0.669 mg/gm liver; $p<0.001$) with 1.4 fold increase when compared to their respective control after 15 days of exposure (Table 5.9). In ethion exposed group, the highest increase in microsomal protein content was shown by *C. punctatus* (4.055±0.236 mg/gm liver; $p<0.05$) with 1.35 fold increase followed by *H. fossilis* (4.136±0.403 mg/gm liver; $p<0.01$) with 1.34 fold increase and *C. batrachus* (4.110±0.563 mg/gm liver; $p<0.05$) with 1.26 fold increase after 15 days of exposure (Table 5.16). In dicofol exposed group, the

highest increase in microsomal protein content was shown by 15 days *C. batrachus* (4.380±1.128 mg/gm liver; $p<0.05$) with 1.4 fold increase followed by *C. punctatus* (4.205±0.450 mg/gm liver; $p<0.01$) with 1.3 fold increase and *H. fossilis* (4.264±0.656 mg/gm liver; $p<0.05$) with 1.2 fold increase after 15 days of exposure (Table 5.23).

A considerable increase in liver weight, microsomal protein and many microsomal enzyme activities can be produced by treating animals for several days with toxins (Orrenius *et al*., 1965; Shuster and Jick, 1966; Kato *et al*., 1968). The increase in protein content in the fish exposed to pesticides suggests that any possible protein loss is compensated by increasing the tissue protein synthesis to meet increased demand to detoxify the pesticide and necessitate enhanced synthesis of detoxification enzyme proteins (Gill *et al*., 1990). Muley *et al*. (1996) also reported an increased level of protein content in gill, muscles and kidney of *Tilapia mosambica* exposed to 0.016 ppb of endosulfan for 168 hours. The increase in microsomal protein content in the present study may be the result of an elevation in tissues metabolic activity induced by pesticides.

H. fossilis treated with deflazacort (0.440±0.096 nmole/mg protein) recorded the highest content of CYP 450 compared to 0.214±0.102 nmole/mg protein in control group (Table 5.1). The CYP 450 content in β-naphthoflavone (β-NF) treated fish, *C. punctatus* (0.432±0.097 nmole/mg protein), *H. fossilis* (0.509±0.069 nmole/mg protein) and *C. batrachus* (0.466±0.118 nmole/mg protein) were also significantly higher ($p<0.001$) compared to their respective control (Table 5.4). The induction of CYP 450 was positively correlated with an elevation in LSI and microsomal protein content after the treatment with β-NF (Table 5.3).

The activities of CYP 450 enzymes was slightly higher in 15 days pesticide treated group compared to 5 days and 10 days treated groups in most of the cases studied in the present work. This could be explained by the increase in protein and CYP P450 content in fish exposed for longer duration (Table 5.9, 5.10, 5.16, 5.17, 5.23 and 5.24). The CYP 450 content after 15 days of exposure to cypermethrin in *C. punctatus*, *H. fossilis* and *C. batrachus* were 0.425±0.053, 0.523±0.234 and 0.484±0.131 nmole/mg protein respectively. These values reflected 1.4, 1.5 and 1.6 fold increase compared to their respective control group (Table 5.10). Of the ethion treated groups, CYP 450 content in *C. punctatus*, *H. fossilis* and *C. batrachus* were 0.550±0.120, 0.599±0.159 and 0.542±0.084 nmole/mg protein respectively and reflected 1.9, 1.84 and 1.83 fold increase compared to their respective control group after 15 days of exposure (Table 5.17). In dicofol treatment, *C. batrachus* (0.705±0.069 nmole/mg protein) showed the highest induction with 2.3 fold increase

followed by. *H. fossilis* (0.713±0.397 nmole/mg protein) with 2.1 fold and *C. punctatus* (0.524±0.031 nmole/mg protein) with 1.8 fold increase when compared with their respective control group after 15 days of exposure (Table 5.24). It has been reported that the enzyme activity is higher if the experimental organism is exposed for a longer duration to the xenobiotics as it brings about the physiological differences within the organism (Arellano-Aguilar *et al.*, 2009).

CYP 450 family of catalytic enzymes are inducible when animals are exposed to toxins and this explains why CYP 450 content was higher in experimental fish exposed to pesticides in comparison to the control. However, CYP 450 proteins only constitute a limited percentage of total microsomal protein content. Another microsomal enzyme system involved in pesticide metabolism is flavin-containing monooxygenase (FMO) (Hodgson *et al.*, 1995). FMO enzyme is also inducible despite the fact that this enzyme is not under the same regulatory control as CYP 450 enzymes (Parkinson, 1996). Thus, the difference of microsomal protein content between control and experimental fish may be the result of induction of both CYP 450 system and FMO system.

The capability of an animal to survive adverse effects of ingested toxins must be considered from the perspective of the potency of the detoxification system. A useful index of the potency of the detoxification system is the amount of enzyme involved in the detoxification (Ling, 2005). Notably, all important activities of the hepatic microsomal detoxification system were considerably higher in experimental fish.

In the present investigation, it was evident that fish have the capacity to increase their detoxifying power when exposed to pesticides. CYP 450 plays a vital role in protecting tissue from oxidative stress and the increase in this enzyme activity in liver indicates the development of a defensive mechanism to counter the effect of pesticides and may reflect the organisms ability to provide more efficient protection against pesticide toxicity. Our studies suggest that pesticide exposed fish may improve their detoxification capacity by enhancing the amount of CYP 450 enzyme system.

Metabolism of pesticides by hepatic cytochrome P450

In this study, all the fish treated with 3 class of pesticides, cypermethrin, ethion and dicofol displayed an elevated level of EROD activity in 5, 10 and 15 days with a significant difference ($p<0.001$), probably due to EROD`s higher detection sensitivity (Table 5.11, 5.18

and 5.25). In cypermethrin treatment, the 15 days treated group showed higher induction than 5 and 10 days treated group compared to the control. The EROD activity after 15 days of exposure to cypermethrin in *C. punctatus*, *H. fossilis* and *C. batrachus* were 99.341±13.451, 97.516±15.615 and 123.934±29.316 pmole resorufin formed/mg protein/min respectively (Table 5.11). Of the ethion treated groups, 5 days exposed *C. punctatus* (99.867±19.907 pmole resorufin formed/mg protein/min) showed the highest induction, while among *H. fossilis*, 15 days (86.944±21.720 pmole resorufin formed/mg protein/min) and among *C. batrachus*, 10 days (111.933±20.613 pmole resorufin formed/mg protein/min) exposed group showed the highest induction (Table 5.18). In dicofol treatment, the 10 days exposed *C. punctatus* (173.690±30.021 pmole resorufin formed/mg protein/min) showed the highest induction with 4.9 fold increase while among *H. fossilis* (153.767±18.840 pmole resorufin formed/mg protein/min) and *C. batrachus* (156.055±8.625 pmole resorufin formed/mg protein/min), the 15 days exposed group showed the highest induction when compared with their respective control group (Table 5.25). Though EROD activity was significantly induced in all the fish species examined, the induction level was of lesser magnitude with ethion exposure compared to cypermethrin and dicofol exposure. This increase in EROD activity proves the important role played by CYP1A isoform.

The main reaction in the metabolism of deltamethrin, cypermethrin or cyhalothrin in rats/mice is the ester cleavage by carboxylesterase (Demoute, 1989). Fish primarily metabolize pyrethroids by oxidative degradation, probably involving hepatic CYP 450-dependent monooxygenase with ester hydrolysis being a secondary reaction (Deer *et al*., 1996). Cypermethrin is metabolized and eliminated significantly more slowly by fish than by mammals or birds (Srivastava *et al*., 2008) compared to ethion and dicofol which may explain this compound's high toxicity in fish compared to other organisms. Although, the evaluation of CYP1A stimulation by EROD activity is a common tool for quantifying environmental exposure to aryl hydrocarbon receptor (AhR) ligands, a number of studies have established CYP1A stimulation in response to pesticides (Haluzova *et al*., 2011). Assis *et al*. (2009) have reported induced level of EROD activity in liver microsomes of *Ancistrus multispinis* in response to deltamethrin exposure. Similar results have also been confirmed in fishes exposed to DDT (Lemaire *et al*., 2010), chlorpyrifos (Rai *et al*., 2010) and dicofol (Pal *et al*., 2011). Somnuek *et al*. (2012) reported chlorpyrifos and carbaryl at high concentrations resulted in significant elevation of CYP1A gene expression. CYP1A subfamily is the most studied CYP 450 isoform and is responsible for a wide range of xenobiotic biotransformation

whose catalytic activity is expressed as the activity of EROD. EROD activity is best viewed as an indicator of contaminant exposure rather than of effect and this biomarker may also serve as a predictive tool for contaminant risk assessment (Popovic *et al*., 2013).

H. fossilis exposed to cypermethrin displayed a significant difference in N, N-dimethylaniline demethylase (N,N-DMA) activity in all the treated groups (5, 10 and 15 days) with the highest elevation seen in 10 days (0.285±0.111 nmole formaldehyde formed/mg protein/min; $p<0.05$) with 2.2 fold increase, while only 15 days treated group displayed a significant difference ($p<0.05$) in *C. punctatus* (0.186±0.082 nmole formaldehyde formed/mg protein/min) with 2.1 fold increase and *C. batrachus* (0.338±0.147 nmole formaldehyde formed/mg protein/min) with 1.6 fold increase in comparison to their respective control (Table 5.12). In ethion treatment, *C. punctatus* revealed negligible variation throughout the 15 days of exposure in N,N-DMA activity and only showed a marginal increase in 15 days treated group when compared to the control group. On the other hand, 5 days (0.212±0.070 nmole formaldehyde formed/mg protein/min; $p<0.05$) and 10 days (0.249±0.077 nmole formaldehyde formed/mg protein/min; $p<0.001$) treated *H. fossilis* and 5 days treated *C. batrachus* (0.312±0.064 nmole formaldehyde formed/mg protein/min; $p<0.05$) displayed a significant difference in comparison to their respective control group (Table 5.19). In case of dicofol treatment, there was a general trend of increase in N,N-DMA activity in all the fish species studied but only 15 days treated *C. punctatus* (0.291±0.091 nmole formaldehyde formed/mg protein/min) and *H. fossilis* (0.267±0.063 nmole formaldehyde formed/mg protein/min) displayed a significant difference ($p<0.001$), whereas, no difference was seen in *C. batrachus* (Table 5.26). The present work demonstrated that the increase in N,N-DMA activity is due to the presence of CYP2B isoform in all the fish species studied.

Earlier studies have reported that phenobarbital, a specific inducer of CYP2B in mammals have not been found to induce CYP2B in fish, however, CYP2B like activities in fish has been reported and suggested that some nutritional or environmental factors might control the expression of these genes in fish (Stegemann *et al*., 1997). It is likely that some CYP 450 differ in their functions among mammals and fish and follow different mechanisms of induction (Price *et al*., 2008) and it may be possible that pesticides do induce CYP2B like activities in fish. The metabolism of organophosphates to their oxon metabolites are likely mediated by CYP1A2, 2B6, 2B1/2, 2D6 and CYP3A4 (Sams *et al*., 2000; Tang *et al*., 2001). CYP2B6 has the highest desulfuration activity, whereas dearylation activity is highest for 2C19. CYP3A4 has high activity for both dearylation and desulfuration (Tang *et al*., 2001).

Rai *et al*. (2010) reported the induction of CYP2B like activities in fish, *Laboe rohita* after exposure to the pesticide, chlorpyrifos. Pal *et al*. (2011) have also reported a significant induction in CYP2B mediated N,N-DMA activity in *C. punctatus* when exposed to 0.2 mg/kg body weight of chlorpyrifos and dicofol. In consequence, whenever CYP 450 enzymes are used as biomarkers in monitoring programs, one should be cautious with metabolic differences, even among fish species. Though specific CYP 450's are necessary to metabolize xenobiotics, pesticides have the ability to induce one or more forms of CYP 450 simultaneously.

C. punctatus treated with cypermethrin displayed no response in aniline hydroxylase (AH) activity, whereas, a significant elevation was seen in 15 days treated *H. fossilis* (0.318±0.152 nmole p-aminophenol formed/mg protein/min; $p<0.05$), and 10 (0.395±0.059 nmole p-aminophenol formed/mg protein/min; $p<0.01$) and 15 days (0.387±0.143 nmole p-aminophenol formed/mg protein/min; $p<0.01$) treated *C. batrachus* when compared with their respective control group. Of the 3 fish species, 10 days treated *C. batrachus* revealed the highest induction with 1.7 fold increase in the activity (Table 5.13). In ethion treatment, only 10 days treated *C. punctatus* (0.177±0.045 nmole p-aminophenol formed/mg protein/min) displayed a significant induction ($p< 0.05$) in the activity compared to their control group, while all the treated groups (5, 10 and 15 days) of *H. fossilis* varied significantly in AH activity with 2.3 fold increase seen after 15 days of exposure (0.455±0.078 nmole p-aminophenol formed/mg protein/min). *C. batrachus* showed no response in AH activity towards ethion treatment when compared to its control, 0.214±0.075 nmole p-aminophenol formed/mg protein/min (Table 5.20). *C. punctatus* showed a marginal decrease in AH activity towards dicofol exposure in all the treated groups (5, 10 and 15 days) compared to the control (0.116±0.051 nmole p-aminophenol formed/mg protein/min) but the value was not significant while in *H. fossilis*, AH activity varied significantly ($p<0.001$). The values reflected a 3.1, 2.6 and 3.8 fold elevation after 5, 10 and 15 days of exposure when compared to its control (0.197±0.082 nmole p-aminophenol formed/mg protein/min). *C. batrachus* showed no response in aniline hydroxylase activity towards dicofol treatment when compared to its control, 0.219±0.079 nmole p-aminophenol formed/mg protein/min (Table 5.27). The present study indicated that the increase in AH activity is due to the presence of CYP2E1 isoform.

CYP2E1 dependent aniline hydroxylase activity has been shown to be a possible way for metabolism in the liver of fish species as in mammals. Although CYP2E1 is responsible for the metabolism of a number of low-molecular-weight xenobiotic compounds, studies have

reported a stimulation of CYP2E1 activity upon exposure to pesticides carbaryl (Tang *et al*., 2002) and parathion (Mutch *et al*., 2003). A study by Sadat *et al*. (2009) reported its role in the metabolism of pesticide with long-term exposure. However, *Labeo rohita* exposed to chlorpyrifos displayed a significant inhibition of CYP2E1 activity (Rai *et al*., 2010). In contrast, Pal *et al*. (2011) reported a significant induction in aniline hydroxylase activity in *C. punctatus* when exposed to pesticides chlorpyrifos and dicofol.

All the treated groups (5, 10 and 15 days) of fish, *C. punctatus*, *H. fossilis* and *C. batrachus* showed a significant inhibition in erythromycin N-demethylase (ERND) activity after exposure to cypermethrin (Table 5.14). The lowest inhibition in *C. punctatus* (0.561±0.139 nmole formaldehyde formed/mg protein/min; $p<0.01$) and *C. batrachus* (0.606±0.108 nmole formaldehyde formed/mg protein/min; $p<0.01$) were shown by 10 days, and in *H. fossilis* by 15 days (0.504±0.142 nmole formaldehyde formed/mg protein/min; $p<0.01$) when compared to their respective control group. The result was in sharp contrast with that of ethion and dicofol treatment where ERND activity was significantly induced (Table 5.21 and 5.28). In ethion treatment, the highest induction was shown by 5 days (1.642±0.612 nmole formaldehyde formed/mg protein/min) exposed group in *C. punctatus* with 1.9 fold increase, by 10 days (3.978±1.067 nmole formaldehyde formed/mg protein/min) exposed group in *H. fossilis* with 4.7 fold increase and by 15 days (1.579±0.520 nmole formaldehyde formed/mg protein/min) exposed group in *C. batrachus* with 1.8 fold increase (Table 5.21). In dicofol treatment, the highest induction in *C. punctatus* (9.566±1.807 nmole formaldehyde formed/mg protein/min) and *C. batrachus* (2.168±0.392 nmole formaldehyde formed/mg protein/min) with 11.2 and 2.4 fold increase was shown by 15 days exposed group, while in *H. fossilis*, 5 days (3.156±1.410 nmole formaldehyde formed/mg protein/min) exposed group with 3.7 fold increase showed the highest induction in ERND activity compared to their respective control group (Table 5.28). The induction and inhibition in ERND activity in the 3 fishes studied proves the vital role played by CYP3A4 isoform.

CYP3A is one of the most abundant CYP 450 isoforms in fish liver possessing a broad range of substrate specificity and accounts for the metabolism of almost 50% of the currently used pharmaceutical agents (Parkinson, 1996; Hegelund and Celander, 2003). Methoxychlor, a structural analogue of the DDT pesticide, is known to induce rat hepatic CYP2B and 3A mRNAs and the corresponding proteins (Blizard *et al*., 2001). It is seen that CYP3A4 is the predominant isoform responsible for metabolizing pyrethroid pesticides in mammals (Scollon *et al*., 2009; Yang *et al*., 2009), thus, a probable explanation for high pyrethroid toxicity in

fish may be due to the inhibition of CYP3A4 isoform in the liver and other tissues. CYP1A, CYP2B and CYP3A4 were also reported to be induced in channel catfish (Stuchal *et al*., 2006) and *Labeo rohita* (Rai *et al*., 2010) after exposure to methoxychlor and chlorpyrifos. However, Pal *et al*. (2011) have reported a marginal decrease in CYP3A4 mediated ERND activity in *C. punctatus* when exposed to chlorpyrifos and dicofol.

The biochemical responses assessed in the present study are the earliest indicators of exposure to stressors that can be detected in an organism. Many biochemical and physiological changes in aquatic organisms are caused by pesticides which influence the activities of several enzymes (Khan and Law, 2005) and pose a long-term risk to mammals, birds, amphibians and fish (Ali *et al*., 2011). Hepatic CYP 450 expression of fish can be induced by exposure to xenobiotics because of the presence of high percentage of CYP 450 in the liver as opposed to other organs.

A great diversity of cytochrome P450 enzymes in fish has been recognized and each isoform of the CYP 450 participates in the metabolism of many different compounds, but one substrate can also be metabolized by several different isoforms (Stegemann and Hahn, 1994; Siroka and Drastichova, 2004). Pesticides are primarily metabolized by the phase I enzymes that include the CYP 450 and flavin monooxygenases, and these enzymes can be either induced or inhibited by the pesticides or their reactive metabolites (Furnes and Schlenk, 2005). Recent studies have identified the presence of CYP1A, CYP2B, CYP2E1, CYP2K1 and CYP3A in the liver of some freshwater fish (Nabb *et al*., 2006) which play a vital role in the detoxification of carbamate and organophosphate insecticides (Ferrari *et al*., 2007).

Most lipophilic chemicals including drugs, carcinogens, pesticides, environmental pollutants and naturally occurring compounds undergo enzyme-mediated oxidative, hydrolytic or conjugative biotransformations in liver and in extrahepatic tissues yielding more polar compounds which can be easily excreted and are useful as early warning indices of environmental alteration, so that remedial measures may be taken by resource protection agency in time to prevent permanent decline in fish populations (Thomas, 1990). The great majority of them are almost devoid of any activity but in certain instances, metabolites are produced that may retain or augment the effects of the parent compounds or even acquire different pharmacological or toxicological properties. Thus, metabolism plays a critical role in determining both the efficacy and the residence time of xenobiotics in the body as well as in modulating the response to toxic chemicals (Akdogan and Sen, 2010).

Thus, the present study demonstrates evidence of CYP 450 related xenobiotic metabolism in the liver of *C. punctatus, H. fossilis* and *C. batrachus* exposed to cypermethrin, ethion and dicofol. This study also established the presence of CYP2B activity which was thought to be absent in fish. The induction of CYP 450 mediated catalytic activity may also serve as a very efficient adaptive strategy of the fish species to increase tolerance to the pesticides and guarantee its survival.

Kinetics of cytochrome P450 mediated metabolism

Vmax and Km are the two parameters that define the kinetic behaviour of an enzyme as a function of substrate concentration. Km is an approximate measure of the affinity of the substrate for the enzyme and Vmax is the maximum rate of an enzyme catalyzed reaction (i.e. when the enzyme is saturated by the substrate) while Vmax/Km ratio acts as an indicator of enzymatic efficiency.

Considering 7-ethoxyresorufin as a substrate for EROD activity, in *C. punctatus* only ethion treated group (0.715±0.073 µM) showed a significantly ($p<0.001$) higher Km value compared to its control (0.421±0.035 µM). *H. fossilis* showed a significantly lower Km value in the entire treated group compared to control (0.731±0.021 µM) with the lowest value seen in ethion treated group (0.477±0.013 µM; ($p<0.001$), while *C. batrachus* showed a significantly ($p<0.001$) higher Km value in the entire treated group when compared to their control group, 0.427±0.019 µM (Table 5.29). This result shows that ethion treatment in *C. punctatus* and all the pesticide, cypermethrin, ethion and dicofol treatment in *C. batrachus* require high substrate concentration to carry out the reaction suggesting a lower binding affinity (high Km value), while in *H. fossilis* all the pesticide, cypermethrin, ethion and dicofol treatment require low substrate concentration to carry out the reaction suggesting a higher binding affinity (low Km value).

Considering N, N- dimethylaniline as a substrate for N,N-DMA activity in *C. punctatus*, all the pesticide treated group showed a significantly lower Km value compared to control (0.285±0.021 mM) with the lowest value seen in ethion treated group (0.165±0.033 mM; $p<0.01$). In *H. fossilis*, the dicofol treated group (0.294±0.020 mM; $p<0.001$) showed higher and cypermethrin treated group (0.156±0.014 mM; $p<0.05$) showed lower Km values compared to its control (0.182±0.013 mM) while in *C. batrachus*, the Km values were homogeneous in all the treated groups with respect to control, 0.244±0.023 mM (Table 5.31).

This result shows that all the pesticide treatment in *C. punctatus* have a higher binding affinity for the substrate while in *H. fossilis*, the dicofol treatment have a lower binding affinity and cypermethrin treatment a higher binding affinity to the substrate.

Considering aniline as a substrate for AH activity in *C. punctatus*, the Km values were almost homogeneous in all the treated groups with respect to control (1.056±0.159 mM). In *H. fossilis*, both the ethion (1.133±0.033 mM; $p<0.05$) and dicofol (0.842±0.045 mM; $p<0.01$) treated group showed a significantly lower Km values when compared to the control (1.834±0.120 mM). In *C. batrachus*, only cypermethrin treated group (1.588±0.087 mM) showed a significantly ($p<0.01$) lower Km value in comparison to its control group, 2.170±0.130 mM (Table 5.33). This result shows that ethion and dicofol treatment in *H. fossilis* and cypermethrin treatment in *C. batrachus* have a higher binding affinity to the substrate.

Considering erythromycin as a substrate for ERND activity in *C. punctatus*, cypermethrin treated group (0.233±0.016 mM) showed a significantly ($p<0.001$) higher Km value and ethion treated group (0.041±0.015 mM) showed a significantly ($p<0.001$) lower Km value in comparison to the control (0.105±0.014 mM). In *H. fossilis*, only cypermethrin treated group (0.210±0.016 mM) showed a significantly ($p<0.01$) higher Km value with respect to its control (0.167±0.019 mM). In *C. batrachus*, the Km values were almost homogeneous in all the pesticide treated groups with respect to its control group, 0.137±0.015 mM (Table 5.35). This result shows that ethion treatment in *C. punctatus* have a higher binding affinity for the substrate, while cypermethrin treatment in *C. punctatus* and *H. fossilis* have a lower binding affinity to the substrate.

In the situation where Km value is high, the substrate would be expected to be poorly metabolized until it reached a certain concentration. However, if the inherent toxicity of such a substrate is high, systemic toxicity can take place before the compound is metabolized at a toxicologically significant rate. Thus, in fishes, compounds that undergo detoxification via the reaction having high Km would be expected to have higher ability to produce a toxic effect than compounds that are detoxified via the pathways having low Km (Ling, 2005).

Examination of the Vmax values for EROD activity revealed a significant difference ($p<0.001$) in all the pesticide treated groups with respect to the control. All the fish, *C. punctatus* (42.785±4.560 pmole resorufin formed/mg protein/min), *H. fossilis* (49.685±3.835 pmole resorufin formed/mg protein/min) and *C. batrachus* (40.490±6.359 pmole resorufin

formed/mg protein/min) displayed a homogeneous range in the control group for EROD activity with the highest Vmax value shown by dicofol treated group followed by cypermethrin and ethion treated group (Table 5.29). In the present study, EROD Vmax values were quite similar among the species and despite statistically significant difference, all values were below 200 pmole resorufin formed/ mg protein/ min/. EROD activity is mainly studied to determine its suitability as a biomarker of pollution. Gonzalez *et al*. (2009) reported similar EROD Vmax value when studying kinetic phase I reaction in eight finfish species.

The Vmax for N,N-DMA activity had a variable range in values for both control and treated groups. All the pesticide treated fish, *C. punctatus*, *H. fossilis* and *C. batrachus* displayed a significant difference in N,N-DMA activity compared to their respective control group. The highest Vmax value in *C. punctatus* was shown by dicofol treated group (0.305±0.019 nmole formaldehyde formed/mg protein/min; $p<0.001$), while in *H. fossilis* (0.289±0.020 nmole formaldehyde formed/mg protein/min; $p<0.001$) and *C. batrachus* (0.357±0.021 nmole formaldehyde formed/mg protein/min; $p<0.001$), the cypermethrin treated group showed the highest Vmax value (Table 5.31).

The Vmax value for aniline hydroxylase activity in *C. punctatus* displayed a significant difference in cypermethrin (0.181±0.010 nmole p-aminophenol formed/mg protein/min; $p<0.001$) and ethion (0.149±0.015 nmole p-aminophenol formed/mg protein/min; $p<0.05$) treated group compared to the control. In *H. fossilis*, the Vmax value was significantly higher ($p<0.001$) in the entire pesticide treated groups compared to the control (0.189±0.019 nmole p-aminophenol formed/mg protein/min) with the highest value shown by dicofol treated group (0.720±0.027 nmole p-aminophenol formed/mg protein/min). In *C. batrachus*, only cypermethrin treated group (0.345±0.029 nmole p-aminophenol formed/mg protein/min) showed a significant difference ($p<0.001$) in comparison to the control, 0.229±0.014 nmole p-aminophenol formed/mg protein/min (Table 5.33).

The Vmax value for ERND activity in all the control groups of fish, *C. punctatus* (0.896±0.071 nmole formaldehyde formed/mg protein/min), *H. fossilis* (0.872±0.033 nmole formaldehyde formed/mg protein/min) and *C. batrachus* (0.842±0.023 nmole formaldehyde formed/mg protein/min) displayed a homogeneous range. The cypermethrin treated group in all the fish, *C. punctatus*, *H. fossilis* and *C. batrachus* displayed a significantly lower ($p<0.05$) Vmax value, while ethion and dicofol treated groups displayed a significantly higher ($p<0.01$) Vmax value in comparison to their respective control group (Table 5.35).

A large variability in catalytic activities of hepatic CYP 450 enzymes (CYP1A, CYP2B, CYP2E1 and CYP3A4) was confirmed in our study. In particular, high variation between and within control, and pesticide treated fish species was noted for all the substrate tested. This can be explained by the fact that the activities of various metabolic pathways examined in our study were comprised of the sum of activities of different CYP 450 isoforms. The differences found in Km or Vmax values for given compounds can eventually lead to different toxic outcomes. Variability of catalytic activity of CYP 450 enzymes is a common characteristic of this enzyme system, and this is mainly associated with a large range of variability in the expression of enzyme protein (Lin and Lu, 2001).

All the fish species treated with cypermethrin was found to be a poor metabolizer in ERND activity (Table 5.35) when compared with other CYP 450 enzyme activities. Since the metabolic capacity of CYP 450 enzyme system was not equal in all the fish species examined, the species tested for this research were divided into two subgroups as poor metabolizers, representing those having lower Vmax values and strong metabolizers, having high Vmax values with respect to the control. As a result of variation in metabolism, the conversion and excretion rate of toxins would be expected to vary among individuals. For the compounds with a high intrinsic clearance, increased enzyme activity will have little effect on hepatic clearance (Labaune, 1989). This is normal for the xenobiotic metabolism catalyzed by cytochrome P450 system (McKinnon and Evans 2000).

In the present study, intrinsic clearance expressed as Vmax/Km ratio for 7-ethoxyresorufin revealed that in *C. punctatus*, the dicofol (337.881±62.318) and cypermethrin (238.687±52.658) treated groups were efficient in intrinsic clearance while in *H. fossilis* and *C. batrachus*, all the pesticide treated groups were efficient in intrinsic clearance when compared to their respective control group (Table 5.30). Vmax/Km ratio for N, N-dimethylaniline revealed that in *C. punctatus*, all the pesticide treated groups were efficient in intrinsic clearance with the highest clearance observed in dicofol treated group (1.511±0.464), while in *H. fossilis* and *C. batrachus*, cypermethrin and ethion treated groups were observed to be efficient in intrinsic clearance compared to their respective control (Table 5.32). Vmax/Km ratio for aniline revealed that in *C. punctatus* (0.170±0.053) and *C. batrachus* (0.217±0.065), only cypermethrin treated group was efficient in intrinsic clearance while in *H. fossilis*, ethion (0.394±0.086) and dicofol (0.857±0.123) treated groups were observed to be efficient in intrinsic clearance compared to their respective control group (Table 5.34). Vmax/Km ratio for erythromycin in all the fish, *C. punctatus*, *H. fossilis* and *C.*

batrachus revealed that ethion and dicofol treated groups were efficient in intrinsic clearance. The Vmax/Km ratio was lower in all cypermethrin treated fish but was not significant when compared to their respective control group (Table 5.36).

Intrinsic clearance expressed as Vmax/Km ratio for 7-ethoxyresorufin was seen to be highest followed by erythromycin, N, N- dimethylaniline and aniline in all the fish species studied. This result clearly demonstrates the vital role played by CYP1A and CYP3A4 isoforms as they are considered to be important isoforms in metabolising the xenobiotics.

Vmax/Km is a pure measure of enzyme activity and is not influenced by other physiological factors of liver clearance. The Vmax/Km ratio is an important parameter that will determine the toxicity of a given compound in systemic clearance (Houston, 1994; Cotreau *et al.*, 2005). To extrapolate the *in vitro* kinetic data to metabolic activity *in vivo*, the concept of intrinsic clearance (Vmax/Km ratio) is very useful and important and therefore, liver microsomal intrinsic clearance values can be scaled and used to predict hepatic clearance (Lin, 1998). For compounds with low intrinsic clearance values, the elimination rate of the compounds depends on enzymatic activity. This means enhancement in hepatic enzyme activity, resulting from the addition of an inducer, will have a more profound effect on clearance of such compounds (Stuchal *et al*., 2006). Conversely, enzyme inhibitors will have the opposite effect (Vaccaro, 2003; Zamaratskaia and Zlabek, 2011). Species that displayed low biotransformation capabilities (low Vmax) may have similar catalytic efficiencies than the ones with high maximum velocities due to a higher binding affinity (low Km) of their enzymatic systems (Gonzalez *et al*., 2009).

The study identified a noteworthy interspecies variation existing in the CYP450-mediated metabolism of substrates including resorufin, N, N-dimethylaniline, aniline and erythromycin and there were also differences in the intrinsic clearance between the control and pesticide exposed fish species for all selected substrates. Therefore, in essence, our data support the principle that animals exposed to toxins increase CYP 450 enzyme and thus acquire resistance.

Cytochrome P450 as environmental monitoring tool

In the present study, we caught fish species from 9 water sources present in and around the tea plantation areas in the Terai region of North Bengal where pesticides are routinely used to control the pest population (Fig. 5.6). Altogether, 5 species of fish was caught with the help

of local fisherman. Major CYP 450 dependent mixed function oxidase activities such as CYP1A mediated EROD activity, CYP2B mediated N,N-DMA activity, CYP2E1 mediated AH activity and CYP3A4 mediated ERND activity were characterized in *Channa punctatus, Channa striatus, Channa gachua, Heteropneustes fossilis* and *Clarias batrachus* for the first time. All the fish species collected from the field displayed variable range of activity than those of the fish acclimatized in the laboratory. The fish collected from different sites also displayed a variable range of activity.

Among all the fishes collected from different sampling sites, *C. batrachus* collected from Lotchka showed the highest LSI value (1.42±0.42) and *C. punctatus* collected from Dangra Dangri showed the lowest LSI value (0.92±0.31). In *C. punctatus*, the fish collected from Magurmari (1.16±0.39) displayed a significant difference ($p<0.05$) in comparison to the fish collected from Dangra Dangri (0.92±0.31), and in *C. batrachus*, the fish collected from Lotchka (1.42±0.42) displayed a significant difference ($p<0.05$) compared to fish collected from Magurmari (1.18±0.41). The LSI values were almost homogeneous in all the groups of *H. fossilis* (Table 5.37). The CYP 450 content was higher in fish collected from Katagaon than that collected from other sites. Overall, *C. batrachus* collected from Katagaon (1.066 nmole/mg protein) showed the highest content of CYP 450 than that for *C. punctatus, C. striatus, C. gachua* and *H. fossilis* (Table 5.38).

Of all the fishes, EROD activity was highest in *C. batrachus* (187±52 pmole resorufin formed/mg protein/min) brought from Magurmari than that collected from other sites and lowest activity was found in *C. gachua* (22±12 pmole resorufin formed/mg protein/min) collected from Deomani (Table 5.39). N,N-DMA activity was found to be highest in *H. fossilis* (0.89±0.43 nmole formaldehyde formed/mg protein/min) collected from Dangra Dangri and lowest in *C. striatus* (0.137 nmole formaldehyde formed/mg protein/min) collected from Katagoan. The fish collected from Dangra Dangri and Ariagoan showed the highest activity than that collected from other sites (Table 5.40). Overall, AH activity was highest in *H. fossilis* (0.409 nmole p-aminophenol formed/mg protein/min) collected from Katagoan while *C. punctatus* (0.08±0.03 nmole p-aminophenol formed/mg protein/min) collected from Ariagoan showed the lowest activity than that collected from other sites (Table 5.41). Of all the fish species, ERND activity was highest in *H. fossilis* (4.13±1.52 nmole formaldehyde formed/mg protein/min) collected from Magurmari and the lowest in *C. punctatus* (0.866 nmole formaldehyde formed/mg protein/min) collected from Katagoan (Table 5.42). These differences in enzyme activities could be ascribed to duration,

physiological status and environmental stress faced by the fish living in different water bodies in different locations. The water bodies of Terai region of North Bengal receive a considerable influx of anthropogenic pollutants especially from agricultural run-off, domestic sewage and tea gardens (Pal *et al*., 2011; Singh *et al*., 2015).

Some of the earliest changes to occur in an organism following exposure to an environmental pollutant occur at the cellular level including induction or inhibition of certain enzyme activity and alterations in genetic material or gene expression as a result of xenobiotic and molecular interactions (El-Shehawi *et al*., 2007; Maier *et al*., 2009; Whatley *et al*., 2010).

CYP1A response measured as EROD activity has already been incorporated into some major monitoring programs such as the National Status and Trends Program in the United States (Collier *et al*., 1992) and North Sea Task Force Monitoring Master Plan of the North Sea Nations in Europe (Goksoyr and Forlin, 1992). In fish, EROD activity with its high sensitivity, specificity, feasibility and simplicity of its measurement have been of advantage in using it as a biomarker (Bucheli and Fent, 1995).

The indiscriminate use of pesticides is considered one of the central factors in changing the environment by causing imbalances in the ecosystem, especially in the aquatic system (Sancho *et al*., 1998). Thus the growth and survival of fish are greatly disturbed by the use of pesticides in or near a body of water (Ewing, 1999). Several studies have shown that most fish sampled from the agricultural areas contained detectable levels of pesticides (Sayeed *et al*., 2003; Singh *et al*., 2015). In Canada, losses among 62 imperilled species were significantly more related to rates of pesticide use and species loss was highest in areas with intensive agriculture (Gibbs *et al*., 2009). Over the last decade, CYP 450 isozymes have been employed as a useful tool for measuring the environmental pollution by pesticides. Although CYP1A was classically recognized as being specifically induced by PAHs and pesticide exposure, it is now known that it is not particularly responsive to one unique class of pollutants. It can be used as a biomarker to monitor the ecological risk of various pollutants in the environment (Whatley *et al*., 2010).

Apart from CYP1A activity, CYP2B, 2E1 and CYP3A4 activity has also been reported to be a useful monitoring tool for assessing the environmental pollution. Boyunegmez (2004) studied 3 different fish, leaping mullet, common sole and annular seabream along the Izmer Bay, Turkey and reported that the area was heavily contaminated with PAH, PCB and other organochlorine type persistent organic precarcinogen/carcinogen chemicals by studying

major CYP 450 dependent mixed function oxidase activities such as ethoxyresorufin O-demethylation and methoxyresorufin O-demethylation (CYP1A), benzphetamine N-demethylation (CYP2B), ethylmorphine N-demethylation (CYP3A) and aniline 4-hydroxylation (CYP2E1). Similarly, the CYP 450 monooxygenase activity (CYP1A and CYP2B) of different populations of Nile tilapia has been used to alert possible human health hazards by organochlorate pollution in the Guanadu River of Brazil, a source of drinking water for Rio de Janeiro (Parente *et al*., 2004). Zhu *et al*. (2006) evaluated CYP 450 activity (CYP1 and CYP2 families) in stripey seaperch that were collected from unpolluted sites and placed in cages along a region where oil was discharged in Western Australia. These authors concluded that CYP 450 isoforms act as potential indicators of environmental pollution by hydrocarbon in fish populations.

Field and laboratory investigations have proved that pesticides are highly toxic to a number of non-target organisms even at very low concentrations (Oudou *et al*., 2004). However, it has been reported that variability of enzymatic activity in the field is higher than in laboratory tests due to factors such as physiological differences in species, migration, gender, period of exposure and concentrations of accumulated pollutants during the life of each organism (Schmitt *et al*., 2005; Hinck *et al*., 2006; Kammann *et al*., 2008). Our study revealed a variable range of CYP 450 activities in all the fish species studied. This may be due to the fact that the factors like gender, age, exposure period and concentration of pollutants were not taken into consideration.

Vindimian *et al*. (1993) reported the induction of EROD activity in fish exposed to a mixture of pesticide runoff from nearby vineyards. These findings indicate the multifarious nature of the fundamental mechanisms by which organisms bear exposure to mixtures of pollutants in their environment. Therefore, it is possible that the toxic nature of pesticide mixture is more complicated than a simple concentration or a response addition model typically used for predicting the probable impact of mixtures in aquatic systems (Broderius *et al*., 1995). Carbamate, pyrethroid, organophosphate and organochlorine pesticides are commonly used for controlling a number of agriculturally important pests, as a result, there is a strong possibility that mixtures of these pesticides co-occur in the environment, and therefore, the combined effects of these pesticides should be considered. Herbicide-insecticide mixtures have also been reported to influence organisms in different ways. Some interactions result in augmenting toxicity while others reduce toxicity (Miota, 2000). Singh *et al*. (2015) have also

reported residues of chlorpyrifos, ethion and dicofol in water, sediment and fish tissue from river Deomoni flowing through the tea gardens of Terai region of West Bengal, India.

The results of the present study suggest that CYP 450 isoforms can be used as a valuable monitoring tool for assessing the pollution of the water bodies contaminated with pesticides of different class that can disrupt the development, reproduction and survival of aquatic organisms.

Interspecies variability in *in vitro* metabolism by cytochrome P450

In spite of observed induction of cytochrome P450 (CYP 450) in 3 fish species in response to pesticide contamination, there were important variations between species concerning the level of enzyme activities. The CYP 450 content and enzyme activities in all the 3 fish studied, *C. punctatus*, *H. fossilis* and *C. batrachus* showed a variable range in β-naphthoflavone treatment (Table 5.4). The response to the pesticide cypermethrin, ethion and dicofol also varied between the fish studied. Among the pesticides in terms of LC_{50} value, *C. punctatus* and *C. batrachus* were observed to be highly sensitive towards cypermethrin followed by ethion and dicofol, while *H. fossilis* was highly sensitive to cypermethrin followed by dicofol and ethion (Table 5.5, 5.6 and 5.7).

The results of the present study demonstrated that the CYP 450 enzyme activities varied differently between and within each pesticide treated fish species. EROD, N,N-DMA and AH activities were involved in cypermethrin metabolism while ERND activity was inhibited and did not catalyze the metabolism of cypermethrin in all the 3 fish species studied (Table 5.11, 5.12, 5.13 and 5.14). In *C. punctatus*, the pesticide, ethion increased EROD, AH and ERND activities while no response was observed in N,N-DMA activity. In *H. fossilis* all the activities viz. EROD, N,N-DMA, AH and ERND were increased by ethion treatment. However, in *C. batrachus*, EROD, N,N-DMA and ERND were induced indicating their role in the metabolism of ethion whereas no response was noted in AH activity (Table 5.18, 5.19, 5.20 and 5.21). EROD and ERND activities, in particular, were induced by the pesticide, dicofol in all the 3 fish species. In *C. punctatus*, N,N-DMA activity was also induced after dicofol treatment while AH activity was moderately inhibited. In *H. fossilis*, similar to ethion treatment, all the activities viz. EROD, N,N-DMA, AH and ERND were induced after dicofol treatment. On the other hand, in *C. batrachus*, N,N-DMA and AH activities were not induced by dicofol treatment (Table 5.25, 5.26, 5.27 and 5.28).

All the 3 fish species displayed a marked difference in maximal velocity (Vmax), binding affinity (Km) and catalytic efficiency (Vmax/Km) between and within species population (Table 5.29, 5.30, 5.31, 5.32, 5.33, 5.34, 5.35 and 5.36).

The fish, *Channa punctatus*, *Channa striatus*, *Channa gachua*, *Heteropneustes fossilis* and *Clarias batrachus* collected from different sites in Terai region of North Bengal displayed a variable range of CYP 450 activities (Table 5.38, 5.39, 5.40, 5.41, and 5.42). All these results establish the interspecies differences in metabolizing the pesticides and environmental xenobiotics by CYP 450 enzymes.

Large interspecies differences are seen in organisms and in different species, the same substrate can be metabolised by different CYP 450 isoforms. There are generally marked differences between the species in CYP 450 enzyme content, activity, and susceptibility. It could explain that the organisms have different physiology, habitats, behaviour and feeding habitats and the ability to accumulate, distribute and metabolize contaminants (Bucheli and Fent, 1995). Basal activities may also vary considerably both among and within species (Whyte *et al*., 2000).

In fish, as in other animal species, there are great differences in CYP 450 catalytic activity among individuals of one population as well as among populations which is related to factors such as species, sex (Navas and Segner 2001), diet or season (Machala *et al*., 1997; Aas *et al*., 2001; Schlenk and Di Giulio 2002; Ruus *et al*., 2002; Jorgensen *et al*., 2002).

In the present study, the intra-individual difference among the population was not carried out as the gender was not taken into consideration. Although, the difference in species was noted when comparing their basal CYP 450 mediated monooxygenase activities as well as their Vmax/Km ratio. One of the most important aspects of CYP 450 enzyme system is that the metabolic capacity of CYP 450 enzyme system is not equal to all members of a population, and as a result, the metabolic conversion and excretion rate of toxins varies among individuals ranging from extremely slow to ultra fast (McKinnon and Evans, 2000) caused by genetic polymorphisms or by inhibition or induction of toxin metabolism (Ling, 2005). Individual variability has been noted in populations of *Fundulus heteroclitus*, with evolutionarily adaptive changes in gene expression thought to account for much of this variation (Oleksiak *et al*., 2005; Crawford and Oleksiak, 2007).

Numerous authors observed differences in species, from which no generalized picture can be obtained. EROD activity was generally higher (3 to 10 fold) in salmonids than in cyprinids

(Monod *et al*., 1988). Lindstrom-Seppa and Oikari (1990) found that bream (*Abramis bramd*) responded well as an indicator, whereas perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) showed lower inducibility. Herbivorous fish may show enhanced basal enzyme activities due to natural inducers (Vindimian *et al*., 1991), such as those present in plants or coral reefs (Vrolijk *et al*., 1993). Conversely, barbel (*Barbus barbus*), a benthic carnivore, was more sensitive to pollution than the herbivorous species nase (*Chondrostoma nasus*) (Vindimian *et al*., 1991). Another carnivorous fish, chub (*Leuciscus cephalus*) has been found to have considerably high enzyme activity (Vindimian *et al*., 1993).

Various mechanisms can be associated with differences between species in response to a xenobiotic, which includes genetic defect in a particular metabolic pathway, differences in the Km and Vmax of specific enzyme, the existence of different isozymes, differences in the ratios of important specific isozymes and differences in the ratio of activities of separate enzyme systems that act together to metabolize a specific xenobiotic (deBethizy and Hayes, 1989; Ling, 2005). Thus, it is possible to trace species differences in xenobiotic metabolism to variations in CYP 450 levels, their inducibilities and the existence of different CYP 450 isoforms of the same protein family or subfamily in various species (Lewis *et al*., 1998; Ling, 2005). Generally, the basic reactions and major metabolites of a xenobiotic are similar among species. However, small differences in metabolism can result in major differences in susceptibility to toxicity.

Electrophoresis

Microsomes are the suspension of membrane protein preparations and are thought to possess CYP 450 and other drug metabolising proteins. A careful analysis of gels in hepatic microsomes from *C. punctatus*, *H. fossilis* and *C. batrachus* led to the identification of protein bands at around 50-56 kDa along with other bands at around 20-29 kDa when stained with N,N,N'N' -Tetramethyl-p-phenylenediamine (TMPD) for visualizing peroxidase activity (Fig. 5.7 a, b and c). The bands at around 50-56 kDa are expected to be that of CYP 450 and the other band could be that of haemoglobin and other heme proteins. A coomassie brilliant blue (CBB) stained gel of the proteins (right panel) demonstrated that the lack of β-mercaptoethanol in the sample buffer is responsible for smearing of the protein bands. The gels were stained for peroxidase activity and then stained for protein. The ability to stain the same gel for peroxidase activity and protein allows one to superimpose the gel scans or

photographs of the two stains to verify which protein staining bands contain heme. Such a comparison is much less precise if made by staining two different SDS gels.

Comparisons of SDS-PAGE of microsomal proteins from control and pesticide treated fish, *C. punctatus*, *H. fossilis* and *C. batrachus* identified a protein band of increased intensity with a molecular weight between 50-56 kDa (Fig. 5.8, 5.9 and 5.10 a, b and c). Similar enhancement was observed in a heme-containing protein as evidenced by heme staining (peroxidase activity) of PAGE gels by TMPD (Fig. 5.7 a, b and c).

The hemeproteins CYP 450 have been previously reported to have H_2O_2-peroxidase activity on SDS polyacrylamide gels using TMPD (Scharf *et al.*, 2000; Miota, 2000). One of the larger families of CYP 450-dependent microsomal monooxygenases are the heme-thiolate membrane-associated proteins with a molecular weight between 45-60 kDa (Miota, 2000). Gel electrophoresis separates proteins based on independent physical characteristics and is a powerful tool for resolving complex mixtures of proteins (Palzkill, 2002). All CYP 450 isoforms possess a common heme cofactor (iron protoporphyrin IX) that is required for binding oxygen and creation of the active oxidant species (Henne *et al.*, 2001).

SDS–PAGE results are in agreement with CYP 450 mediated monooxygenase assays viz. CYP1A, CYP2B, CYP2E1 and CYP3A4 presented in the results section (5.5, 5.6 and 5.7), although some of the activities displayed a negligible response in experimental groups (Table 5.10, 5.11, 5.12, 5.13, 5.14, 5.17, 5.18, 5.19, 5.20, 5.21, 5.24, 5.25, 5.26, 5.27 and 5.28). The band intensities, however, appear to directly correspond with CYP 450 monooxygenase activity results. Together, these findings suggest that potential CYP 450 isoforms are constitutively overexpressed in pesticide resistant populations, while induction of various forms is greater for more susceptible populations. Multiple CYP 450 forms are clearly present, possibly several in a given heme-stained band, the isolation of distinct electrophoretic forms will be necessary to assist future studies of CYP 450 in this species.

Gene sequencing and Phylogenetic analysis

The CYP1A genomic sequence for *H. fossilis* with 534 bp coding 146 amino acid and *C. batrachus* with 509 bp coding 137 amino acid submitted by us is the first report of a partial genomic sequence of any CYP 450 gene of *H. fossilis* and *C. batrachus* (Fig. 5.12 and 5.13), while the cDNA sequence for *C. punctatus* have been previously submitted in the Genbank (Raisuddin and Lee, 2008; EU930319). The studies of xenobiotic metabolizing genes in

Siluriformes fish have attracted lesser attention as compared to fish from other orders (Kim *et al*., 2008). Since *C. punctatus*, *H. fossilis* and *C. batrachus* is commercially and ecologically important fish in various parts of Asia, its genomics may provide impulsion for its sustainable commercial exploitation and preservation and its relationship with other species.

C. punctatus CYP1A structural gene (2511 bp) contained 6 exons and 5 introns while *H. fossilis* (534 bp) and *C. batrachus* (509 bp) contained 2 exons and 1 intron (Table 5.43). In *C. punctatus,* no differences were found between the CYP1A cDNA and exons of the CYP1A genomic DNA obtained in the present study. The large structural gene of *C. punctatus* (2511 bp) is due to the fact that 3 different sequences of genomic DNA amplified by using 3 different primers were attached together to make it a single structural genomic DNA (Fig. 5.11). All the introns begin with the sequence GT and end with AG, consistent with the GT/AG rule of exon-intron junction sequences (Hassanin *et al*., 2009). The phylogenetic tree based on the amino acid sequences clearly shows *C. punctatus* and *C. maculata* CYP1A and *H. fossilis* and *C. batrachus* CYP1A to be more closely related to each other than to other CYP1A subfamilies with 93% identity. *H. fossilis* CYP1A with 86% identity and *C. batrachus* CYP1A with 85% identity were more closely related to *Peltobagrus fulvidraco*, another member of the order Siluriformes (Table 5.44 and Fig. 5.14).

The CYP1A isoform is only considered for gene sequencing in the present study due to its high sensitivity towards a vast majority of xenobiotics compared to other isoforms. The position of the ancestral CYP1A locus remains a question and opposing orientations of CYP1A in pufferfish and frog genes pose a dilemma in ascertaining whether CYP1A1 or CYP1A2 resides in the ancestral CYP1A gene locus (Goldstone and Stegemann, 2006). It remains confusing and somewhat arbitrary and accordingly, in fish, CYP1A is not being provided with a number following the subfamily.

The CYP1A gene subfamily comprises a single ancestral representative of most fish species and two representatives in higher vertebrates (Kimura *et al*., 1984; Gilday *et al*., 1996; Morrison *et al*., 1998). Fish CYP1A is thought to be a hybrid protein coded by a gene ancestral to both mammalian CYP1A1 and CYP1A2 forms, therefore, the name CYP1A rather than CYP1A1 has been suggested (Stegemann *et al*., 1997). Although CYP1A seems to be well conserved across vertebrate taxa (Goldstone *et al*., 2007), levels and inducibility of CYP1A protein and catalytic activity exhibit a rather large variability between fish species and populations (Parente *et al*., 2009).

Liver CYP1A induction in fish by certain classes of chemicals has been widely applied as a biomarker in field studies. The cloning and sequencing of CYP1A gene have been conducted from many fish species for use in assessing the contamination of the aquatic environment (Meyer *et al*., 2002; Fent, 2003; Moore *et al*., 2003; Kim *et al*., 2008). Pesticides like chlorpyrifos and carbaryl were also found to increase CYP1A both on protein and gene levels and the induction was characterized by a dose-dependent increase of EROD activity, which was correlated well with CYP1A mRNA levels in liver (Somnuek *et al*., 2012). Members of subfamily CYP1A are involved in the metabolism of environmental pollutants leading to its detoxification or bioactivation. Understanding the functional evolution of these genes is indispensable for predicting and interpreting species differences in sensitivity to toxicity by environmental pollutants.

From the above discussion and results of the experiments, it can be concluded that CYP 450 isoforms (CYP1A, CYP2B, CYP2E1 and CYP3A4) fulfill the criteria to be used as biomarkers in environmental monitoring. It can also be inferred that the induction of CYP 450 isoforms may be a defensive mechanism, whereby the cell can detoxify potentially lethal xenobiotic compounds that might otherwise accumulate. This knowledge of the mechanisms of induction may provide insights into the strategies that organisms use to adapt to a changing environment and provide a better understanding of the biochemical pathways which control, respond and recognize the chemical stimuli occurring within the cell.