4. MATERIALS AND METHODS

4.1. Experimental animal

Channa punctatus, *Heteropneustes fossilis* and *Clarias batrachus* were choosen as the experimental animal for this research work (Fig. 4.1, 4.2 and 4.3). These 3 fish species are native to South and South-east Asian countries (Froese and Pauly, 2011; Debnath, 2011; Khan *et al.*, 2012). They are freshwater air breathing teleost fish with accessory respiratory organ and one of the most important fish species of flood plains. They are abundantly found in ponds, swamps, beels and canals generally in the plains. They prefer stagnant waters in muddy streams and rivers. They have a great demand in the market because of its high nutritional value.

Figure 4.1. *Channa punctatus.*
Common name: Spotted snakehead
Local name: Lata,taki,okol

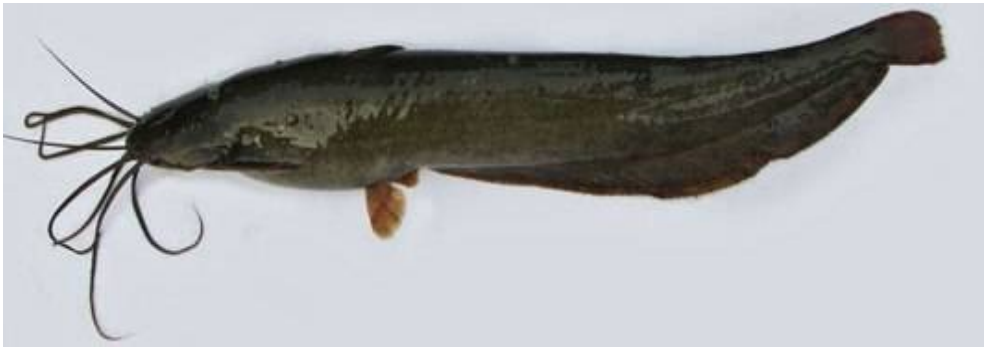

Figure 4.2. *Heteropneustes fossilis.*
Common name: Stinging catfish
Local name: Singhi

Figure 4.3. *Clarias batrachus.*

Common name: Walking catfish

Local name: Magur

4.2. Collection and maintenance of healthy fish in the laboratory

Healthy fish, *Channa punctatus*, *Heteropneustes fossilis* and *Clarias batrachus* weighing 30±5 gm (body length 8-12 cm) were collected and after a bath in 0.1% $KMnO_4$ solution for 1 min to avoid any infection, the fishes were kept in glass aquaria measuring 90x35x35 cm and the depth of water were maintained at 20 cm. The supplier provided the fish caught from areas with no record of pesticide contamination. The fishes were acclimatized for a period of two weeks under laboratory conditions before the start of the experiment. They were fed regularly with small pieces of chopped fish at fixed rate during the course of the experiment.

4.3. Collection of healthy fish from the field

4.3.1. Sample collection

A survey was done to identify the resident fishes in the rivers and ponds flowing through the tea plantation areas of Terai region of North Bengal. Fishes were collected during November, 2012 to June, 2013 using cast net and brought to the laboratory in live condition and immediately sacrificed for microsome preparation.

4.3.2. Sampling stations/sites

Nine sampling sites (Site 1-Site 9) were selected based on the criterion of easy accessibility, proximity to conventional tea gardens and abundance of local fish species (Fig. 4.4 and 4.5). The sampling was done between 2012 and 2013 during pre- and post-monsoon season.

Site 1: Pond in Balarampur, Chopra (26.351855N, 88.365612E)

Site 2: Pond in Katagaon, Chopra (26.354489N, 88.384723E)

Site 3: Pond in Dangra Dangri, Kalagach (26.439045N, 88.338341E)

Site 4: Pond in Ariagoan, Kalagach (26.437831N, 88.359505E)

Site 5: Pond in Jaikhori, Sonapur (26.424007N, 88.269311E)

Site 6: Magurmari river, NBU (26.712514N, 88.355991E)

Site 7: Lotchka river, NBU (26.713385N, 88.343561E)

Site 8: Changa river (26.69131N, 88.25442E)

Site 9: Deomani river (26.69231N, 88.26339E)

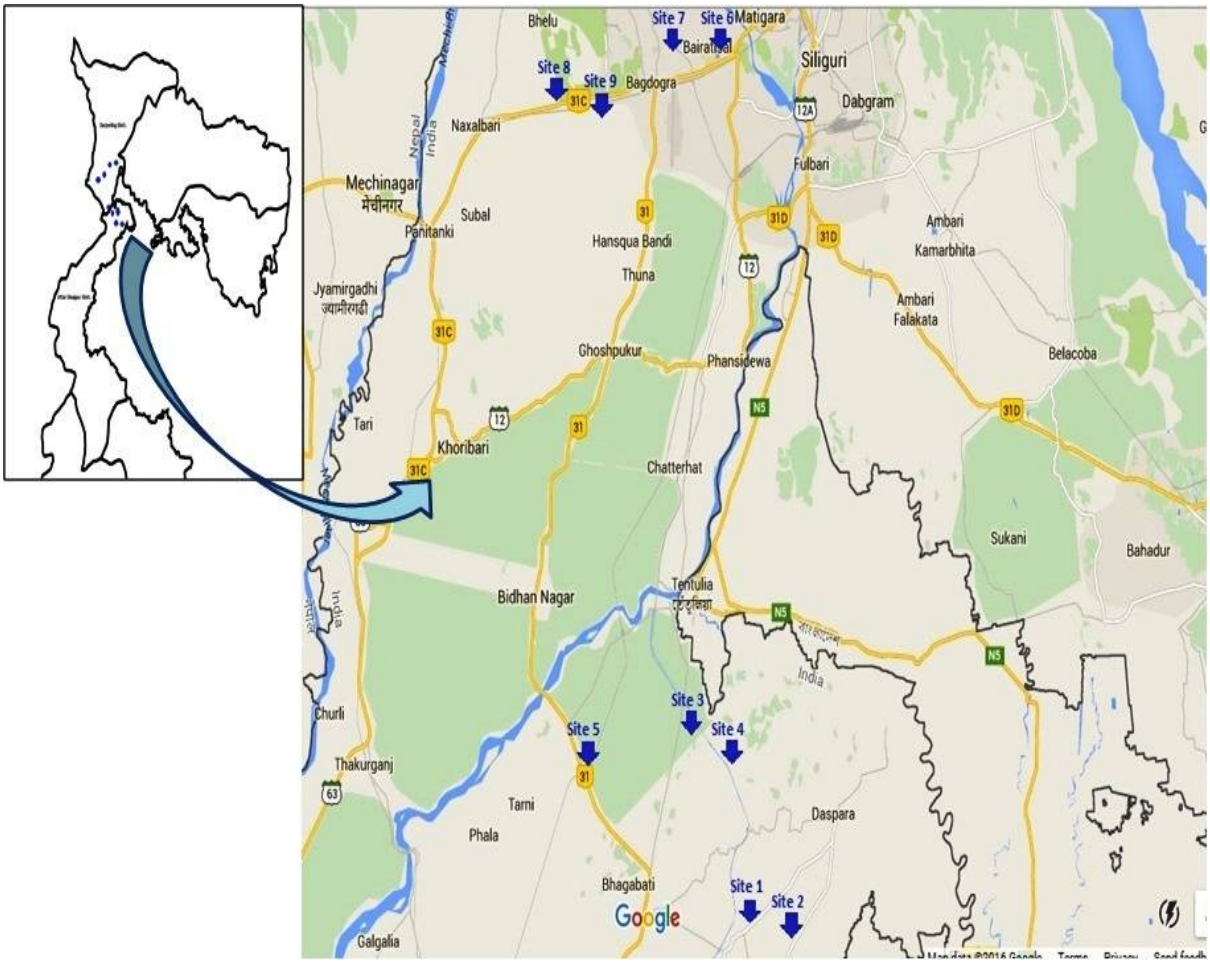

Figure 4.4. Map showing sampling stations in Terai region of North Bengal.

Site 1: Pond in Balarampur

Site 2: Pond in Katagaon

Site 3: Pond in Dangra Dangri

Site 4: Pond in Ariagoan

Site 5: Pond in Jaikhori, Sonapur

Site 6: Magurmari river, NBU

Site 7: Lotchka river, NBU

Site 8: Changa river

Site 9: Deomani river

Figure 4.5. Sampling sites in the Terai region of North Bengal.

4.4. Treatment of the fish with mammalian specific inducers of CYP 450 isoforms

Fish, *Heteropneustes fossilis* were given a single intraperitoneal (i.p.) injection of 20 mg/kg bodyweight of naphthalene, phenobarbitone and deflazacort and 2 ml/kg body weight of acetone. The inducers were dissolved in sesame oil and the control group was administered with only sesame oil. The fish were anaesthetized by dipping the fish in eugenol (0.03%) water. Once the animal was in a state of deep anaesthesia, the required dose of i.p. injection was administered. After an exposure of 72 hours, the enzyme activities were studied with respect to control groups.

4.5. Treatment of the fish with CYP1A specific inducer β-naphthoflavone

Fish, *Channa punctatus*, *Heteropneustes fossilis* and *Clarias batrachus* were treated by i.p. injections of soyabean oil containing 50 mg/kg body weight doses of β- naphthoflavone (β-NF). Control fish received soyabean oil alone. The fishes were anaesthetized by dipping the fish in eugenol (0.03%) water. Once the animal was in a state of deep anaesthesia, the required dose of i.p. injection was administered.

4.6. Determination of LC_{50} value of pesticides

Reagents/Pesticides

- **Cypermethrin (Ripcord) 20 µg/ml Stock Solution:** 0.1 ml cypermethrin was dissolved in 49.9 ml distilled water.
- **Ethion 100 µg/ml Stock Solution:** 0.1 ml ethion was dissolved in 49.9 ml distilled water.
- **Dicofol (Colonel-S) 44 µg/ml Stock Solution:** 0.1 ml dicofol was dissolved in 49.9 ml distilled water.

Probit analysis (Finney, 1971) was adopted to calculate the LC_{50} value. Initially, all the 3 fish species, *C. punctatus, H. fossilis* and *C. batrachus* were kept in a large glass aquarium separately for acclimatization. Thereafter, the fish were separated in a group of 10 fish per aquarium. A total of 50 fish were taken for conducting a single experiment. The fish were administered with 5 different concentrations of the pesticide, cypermethrin (Ripcord 10% EC), ethion (ethion 50 % EC) and dicofol (COLONEL S 18.5 % EC) while the control fish

received no pesticide application (Fig. 4.6). The range of the pesticide concentration varied in all the experimental fish. The mortality was recorded for 24, 48, 72 and 96 hours. The fishes were not provided with food during the course of an experiment. The lethal concentration – 50% end point (LC_{50}) was calculated from the relationship between the probits of percentage mortalities and the logs of concentration of the pesticide application.

a b c

Figure 4.6. Pesticide formulations: (a) Cypermethrin, (b) Ethion, and (c) Dicofol.

4.7. Treatment of the fish with 1/3 sub-lethal concentration of the pesticides, Cypermethrin 10% EC (RIPCORD), Ethion 50 % EC and Dicofol 18.5 % EC (COLONEL S)

10 fishes were randomly taken in a group each for control and treated. A 1/3 sub-lethal concentration of the 96 hour LC_{50} value of the pesticides, cypermethrin, ethion and dicofol based on acute toxicity data generated from the laboratory experiments was used to treat the experimental fish. The water was renewed every 48 hours with a fresh pesticide for the treated groups and only water for the control group. Homogeneity was maintained in all the groups by providing similar experimental conditions.

4.8. Surgery

The fish were killed by a knock on the head and a midline incision was made along the abdomen. The gastrointestinal tract was retracted and the liver was excised using blunt-ended scissors, taking care not to break the bile duct (Fig. 4.7. a and b).

4.9. Liver Somatic Index (LSI)

Livers were excised, weighed and the liver somatic index was calculated as the percentage ratio of liver weight to body weight. The LSI was calculated by using the formula:

LSI = (liver weight/total live weight) X 100.

Since the liver samples were too small to be processed independently for enzyme activity, they were pooled before homogenization.

4.10. Preparation of microsomes

Reagents

- **Perfusion buffer (1.15% KCl, 1mM EDTA, pH 7.4):** 5.75 gm KCl and 0.186 gm EDTA was dissolved in 450 ml distilled water. The pH was adjusted to 7.4 using HCl and the final volume was maintained at 500 ml.
- **Homogenization buffer (1.15% KCl, 1mM EDTA and 0.05M Tris, pH 7.4):** 1.15gm KCl, 0.037 gm EDTA and 0.605 gm Tris were dissolved in 80 ml distilled water. The pH was adjusted to 7.4 using HCl and the final volume was maintained at 100 ml.
- **Resuspension buffer (0.05M Tris, 1mM EDTA and 20% Glycerol, pH 7.4):** 0.605 gm Tris, 0.037 gm EDTA and 20 ml glycerol were dissolved in 80 ml distilled water. The pH was adjusted to 7.4 using HCl and the final volume was maintained at 100 ml.

The procedure for preparation of microsomes was based on the method reported by Chang and Waxman (1998) with minor modifications (Fig. 4.8). Livers were perfused with a large volume of ice cold perfusion buffer (1.15% KCl, 1mM EDTA, pH 7.4) to get rid of unwanted tissues, fat bodies and blood and homogenized (1.15% KCl, 1mM EDTA and 0.05M Tris, pH 7.4) using a glass-teflon homogenizer at 4°C (4 ml per gram of tissue). The homogenized

tissues were centrifuged in a cooling centrifuge (Sigma 3K30) at 12,000 g for 20 minutes at a temperature of 4°C. The supernatant (post-mitochondrial supernatant, PMS) were carefully harvested, without disturbing the pellet and further centrifuged in a super speed vacuum centrifuge (VS35SMTi) at 1,00,000 g for 60 minutes at 4ºC. The supernatant was discarded (cytosol fraction) and the microsomal pellet was resuspended in a resuspension buffer containing 0.05M Tris, 1mM EDTA and 20% Glycerol, pH 7.4 (2 ml per gm of original tissue weight). The fraction was maintained on ice for immediate analysis or stored at -20°C for future use.

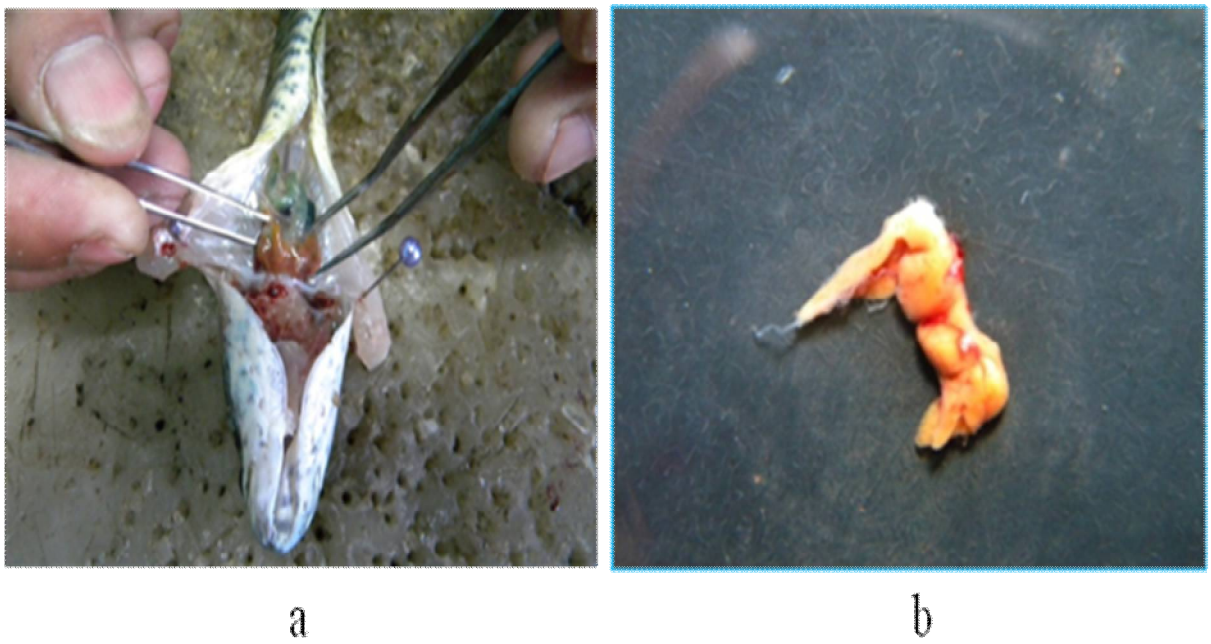

Figure 4.7. (a) Dissection of fish (b) Dissected out liver.

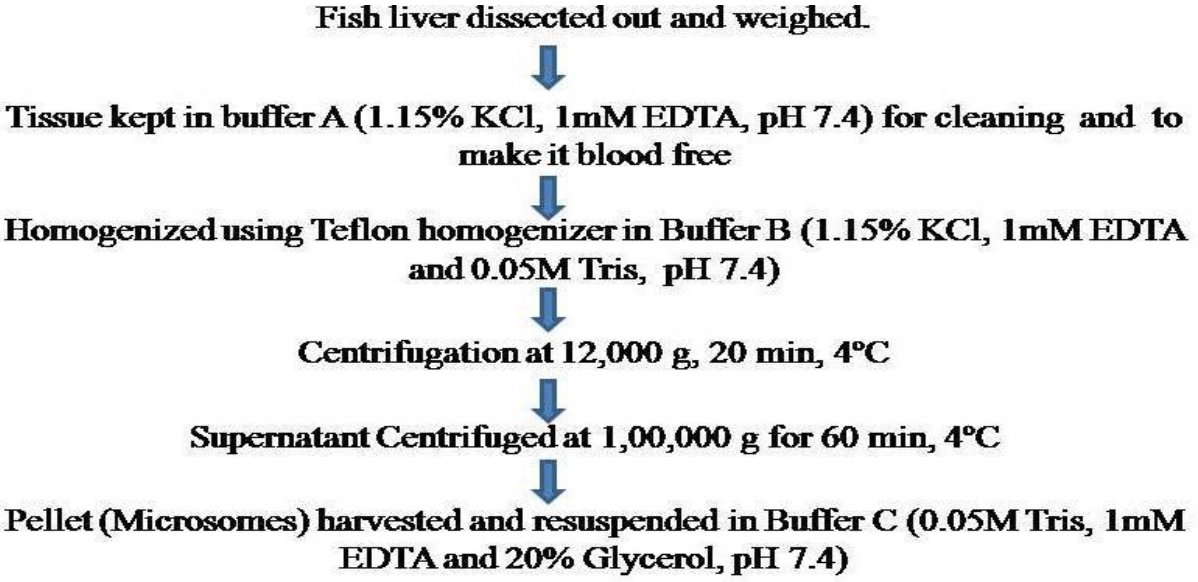

Figure 4.8. Schematic representation for the preparation of microsomes.

4.11. Protein estimation

Reagents

- **0.1 M Sodium hydroxide (NaOH):** 0.4 gm NaOH was dissolved in 100 ml distilled water.
- **Solution A:** 2% Na_2CO_3 was dissolved in 0.1M NaOH (2g of Na_2CO_3 in 100ml of 0.1M NaOH)
- **Solution B:** 1% $CuSO_4$ and 2% sodium potassium tartrate was dissolved in 100 ml distilled water.
- **1N Folin - Ciocalteu reagent:** Folin - Ciocalteu was diluted [1:1] with distilled water from a 2N commercial preparation and made fresh daily.
- **Bovine Serum Albumin (BSA):** 1 mg BSA was dissolved in 1 ml distilled water.

Protein in the microsomal fraction was estimated as described by Lowry *et al.* (1951). 2 gm sodium carbonate (Na_2CO_3) was dissolved in 0.1 M sodium hydroxide (NaOH)- (Solution A). 1% copper sulphate and 2% sodium potassium tartarate were prepared by mixing together in 100 ml distilled water (Solution B). A fresh alkaline solution was then prepared by mixing Solution A and Solution B in a ratio of 50:1 (A:B). In a test tube, 20 µL microsomal protein was diluted with 80 µL distilled water to make the volume 1 ml and to it 5 ml alkaline solution and 1 N 0.5 ml folin-ciocalteau reagent was added. The mixture was then incubated

at room temperature for 30 minutes and the resulting blue colour intensity was measured at 660 nm. The standard curve of BSA from 20 to 100 μg was plotted and used for determination of protein concentration of samples.

4.12. Spectral analysis and quantification of cytochrome P450

Reagents

- **0.2 M Potassium Phosphate Buffer (pH 7.4):** 2.721 gm KH_2PO_4 and 3.483 gm K_2HPO_4 was dissolved independently in 100 ml distilled water. Then 19 ml of KH_2PO_4 and 81 ml of K_2HPO_4 mixed together to get a pH 7.4.
- **Sulfuric acid** and **Formic acid** for the preparation of carbon monoxide gas.
- **Potassium hydroxide** solution
- **Sodium dithionite**

Spectral analysis of cytochrome P450 in the hepatic microsomal fraction was done using the method described by Omura and Sato (1964) with minor modification. The microsomal preparation was diluted in 0.1M phosphate buffer and saturated with carbon monoxide for 40-60 seconds. Carbon monoxide was prepared in a glass bottle by mixing sulfuric acid and formic acid and then passed to the cuvette through another bottle having a solution of potassium hydroxide (Fig. 4.9).

The sample was equally divided between two cuvettes. The baseline (400nm to 500nm) was recorded following which only one of the cuvette was mixed with a few mg of sodium dithionite in order to reduce CYP 450 (Table 4.1) and the absorbance was measured in a spectrophotometer (RAY LEIGH, UV-2601). The data was used to construct a graph using Microsoft office excel 2007.

The CYP 450 content was determined by using the extinction coefficient (ΔE450-490) of 91 mM^{-1} cm^{-1}.

Figure 4.9. A carbon monoxide preparing apparatus.

Table 4.1. Total CYP 450 assay mixture for a final volume of 4mL.

Potassium Phosphate Buffer (pH 7.4)	3.5 mL
Microsomes	0.5 mL
Sodium Dithionite	Few mg

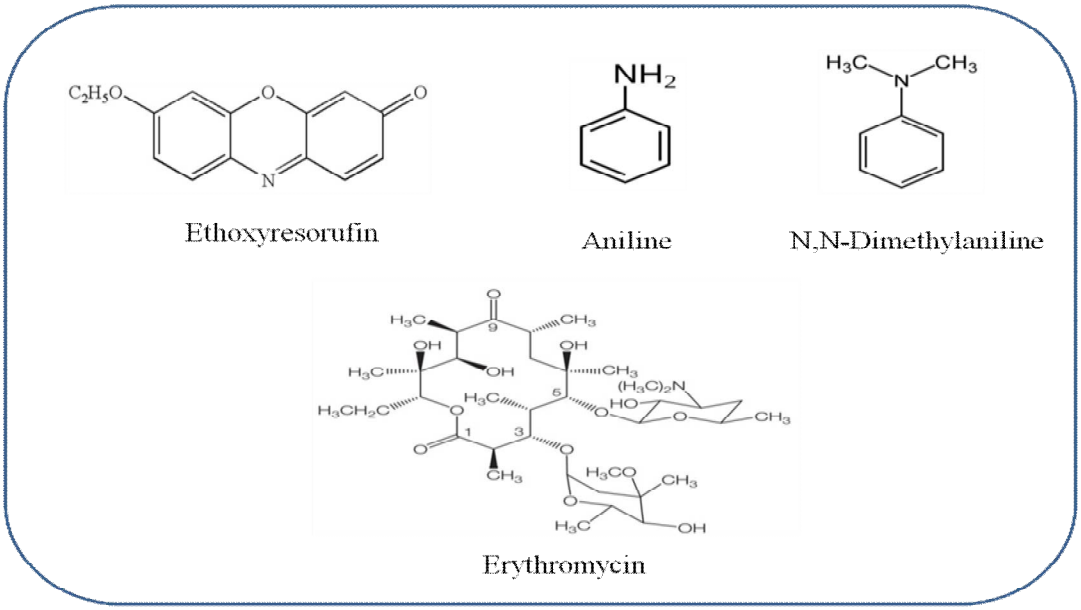

Figure 4.10. Chemical structures of substrates 7-ethoxyresorufin, aniline, N, N-dimethylaniline and erythromycin.

Chemical structures of substrates selected for the study of major detoxification reactions including O-deethylation using 7- ethoxyresorufin as probe substrate, aromatic hydroxylation using aniline as probe substrates and N-demethylation using N, N-dimethylaniline and erythromycin as probe substrates are shown in Fig. 4.10.

4.13. Enzyme assay

The methods CYP1A mediated EROD, CYP2B mediated N, N-dimethylaniline demethylation (N,N-DMA), CYP2E1 mediated aniline hydroxylation (AH) and CYP3A4 mediated erythromycin N-demethylation (ERND) assay were standardized to determine the activities of microsomal mixed function oxidase (CYP 450).

4.13.1. Determination of EROD (ethoxyresorufin-O-deethylase) activity

Reagents

- **0.1 M Tris buffer:** 1.2114 gm Tris-HCl was dissolved in 80 ml distilled water and the pH was adjusted to 7.8. The final volume was maintained at 100 ml.
- **0.1 M NaCl:** 0.588 gm NaCl was dissolved in 100 ml distilled water.
- **7-Ethoxyresorufin (2 µM):** 25 µl of 124 µM stock solution of 7-Ethoxyresorufin was dissolved in 1.5 ml of the reaction mixture to get a solution of 2µM.

- **0.01 M NADPH:** 8 mg NADPH was dissolved in 1 ml of 1% $NaHCO_3$ (1 gm in 100 ml).
- **20% TCA:** 20 gm TCA was dissolved in 100 ml distilled water.

CYP1A dependent EROD (7-Ethoxyresorufin O-deethylase) activity of fish liver microsomes was determined by the spectrophotometric method of Klotz *et al*. (1984) with minor modifications. The reaction mixture of 1.5 ml consisted of 1.35 ml (0.1 M) tris buffer-pH 7.8, 25 µL (0.1 M) NaCl, 25 µL (2 µM) 7-ethoxresorufin, and 50 µL of microsomes. After pre-incubation at 32°C for 5 minutes, the reaction was initiated by the addition of 50 µL (0.01 M) NADPH and the reaction was stopped following 10 minutes of incubation at the same temperature with 0.5 ml of ice-cold trichloroacetic acid (TCA) and centrifuged at 20,000 g for 10 min. The resulting brown colour of resorufin formed in the supernatant was measured at 572 nm.

Resorufin solution was used as a standard. 4 different standard concentrations of resorufin (50, 100, 150 and 200 pmoles) and other incubation constituents were run under the same conditions as for reaction mixture for constructing a standard calibration curve and used for calculation of enzyme activities.

4.13.2. Determination of N, N-dimethylaniline demethylase (N,N-DMA) activity

Reagents

- **0.1 M Na-K Buffer (pH 7.4):** 1.56 gm NaH_2PO_4 and 3.483 gm K_2HPO_4 was dissolved separately in 100 ml distilled water. Then 19 ml of NaH_2PO_4 and 81ml of K_2HPO_4 was mixed together to get a pH 7.4.
- **0.15 M $MgCl_2$:** 0.305 gm $MgCl_2$ was dissolved in 10 ml distilled water.
- **0.01 M N,N-DMA:** 0.012 ml N, N-Dimethylaniline was dissolved in 10 ml acetone.
- **0.1 M Semicarbazide:** 0.111 gm Semicarbazide was dissolved in 10 ml distilled water.
- **0.01 M NADPH:** 8 mg NADPH was dissolved in 1 ml of 1% $NaHCO_3$ (1 gm in 100 ml).
- **25% $ZnSO_4$:** 25 gm $ZnSO_4$ was dissolved in 100 ml distilled water.
- **Saturated $Ba(OH)_2$:** $Ba(OH)_2$ was dissolved in 100 ml distilled water and allowed to settle down. Then the clear solution was filtered through Watman paper.

- **Nash Reagent:** 15 gm ammonium acetate, 0.3 ml acetic acid and 0.2 ml acetylacetone was mixed together in a final volume of 50 ml.

CYP2B dependent N,N-DMA assay was determined by the method of Schenkman *et al*. (1967) with minor modifications. The reaction mixture of 1 ml consisted of 300 µL (0.1 M) Na-K buffer-pH 7.4, 70 µL (0.15 M) $MgCl_2$, 30 µL (0.1 M) semicarbazide, 50 µL (0.01 M) N, N-dimethylaniline and 500 µL microsomes. The mixture was incubated for 5 minutes at 32°C and the reaction started by adding 50 µL (0.01 M) NADPH instead of NADPH-generating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $MgCl_2$). Following aerobic incubation for another 30 minutes, the reaction was terminated by adding 0.5 ml each of 25% zinc sulfate and saturated barium hydroxide. After centrifugation at 10,000 g for 10 min, 1 ml of the supernatant was mixed with 2 ml of double strength Nash reagent and incubated at 60°C for 30 min. The yellow colour of formaldehyde formed as the end product of N,N-DMA activity was measured by the method of Nash (1953) at 412 nm.

A freshly prepared formaldehyde solution was used as a standard. The tubes contained 5 standard concentrations (20, 40, 60, 80 and 100 nmoles) as well as other incubation constituents mixed by Nash reagent and incubated at 60°C for 30 minutes to give the same colour reaction. A standard formaldehyde calibration curve was constructed and used for calculation of enzyme activities.

4.13.3. Determination of aniline hydroxylase (AH) activity

Reagents

- **0.12 M Tris buffer:** 1.453 gm Tris-HCl was dissolved in 80 ml distilled water and the pH was adjusted to 7.4. The final volume was maintained at 100 ml.
- **0.1 M $MgCl_2$:** 0.203 gm $MgCl_2$ was dissolved in 10 ml distilled water.
- **0.1 M Aniline:** 0.091 ml aniline was dissolved in 10 ml distilled water.
- **0.01 M NADPH:** 8 mg NADPH was dissolved in 1 ml of 1% $NaHCO_3$ (1 gm in 100 ml).
- **20% TCA:** 20 gm TCA was dissolved in 100 ml distilled water.
- **1 M Sodium carbonate:** 10.6 gm Na_2CO_3 was dissolved in 100 ml distilled water.

- **Phenol reagent (2% phenol in 0.2 N NaOH):** 0.8 gm NaOH was dissolved in 100 ml distilled water to get 0.2 N NaOH. Then 2 ml saturated phenol was dissolved in 98 ml of 0.2 N NaOH to get a solution of phenol reagent.

CYP2E1 dependent aniline hydroxylase assay was carried out by the procedure of Schenkman *et al*. (1967) with minor modifications. The reaction mixture of 1 ml consisted of 250 µL (0.12 M) tris-pH 7.4, 100 µL (0.1 M) $MgCl_2$, 100 µL (0.1 M) aniline and 500 µL microsomes. The temperature of thereaction mixture was raised to 32°C by incubation in a water bath for 5 minutes and the reaction was started by adding 50 µL (0.01 M) NADPH. The reaction was terminated after 30 minutes by adding 0.5 ml of ice-cold 20% trichloroacetic acid. The precipitate was removed by centrifugation at 10,000 g for 10 min and 1 ml of supernatant was added to 1 ml of a solution containing 2% phenol in (0.2 M) NaOH and 1 ml of (1 M) Na_2CO_3. After 30 min of incubation at 32°C, the blue colour of p-aminophenol formed as the end product of aniline hydroxylase activity was measured at 630 nm.

The p-aminophenol solution was used as a standard. 6 different standard concentrations of p-aminophenol (5, 10, 15, 20, 25 and 30 nmoles) and other incubation constituents were run under the same conditions as for reaction mixture. A standard calibration curve was constructed and used for calculation of enzyme activities.

4.13.4. Determination of erythromycin N-demethylase (ERND) activity

Reagents

- **0.05 M Potassium Phosphate Buffer (pH 7.4):** 0.680 gm KH_2PO_4 and 0.870 gm K_2HPO_4 was dissolved independently in 100 ml distilled water. Then 28 ml of KH_2PO_4 and 72 ml of K_2HPO_4 was mixed together to get a pH 7.2.
- **0.15 M $MgCl_2$:** 0.305 gm $MgCl_2$ was dissolved in 10 ml distilled water.
- **0.01 M Erythromycin:** 0.073 gm erythromycin was dissolved in 10 ml acetone.
- **0.01 M NADPH:** 8 mg NADPH was dissolved in 1 ml of 1% $NaHCO_3$ (1 gm in 100 ml).
- **20% TCA:** 20 gm TCA was dissolved in 100 ml distilled water.
- **Nash Reagent:** 15 gm ammonium acetate, 0.3 ml acetic acid and 0.2 ml acetylacetone was mixed together in a final volume of 50 ml.

CYP3A4 dependent erythromycin N-demethylase assay was carried out following the method of Werringloer (1978). The reaction mixture of 1 ml consisted of 250 µL (0.05 M) phosphate buffer-pH 7.25, 100 µL (0.15 M) $MgCl_2$, 100 µL (0.01 M) erythromycin and 500 µL microsomes. Reactions were initiated by the addition of 50 µL (0.01 M) NADPH and the mixture were incubated for 10 min at 32°C. The reaction was terminated by adding 0.5 ml of 20% trichloroacetic acid. Precipitated proteins were removed by centrifugation at 10,000 g for 10 min. 1 ml of supernatant were added to 1 ml of Nash reagent and incubated at 60°C for 30 minutes. The yellow colour of formaldehyde formed as the end product was measured at 412 nm.

The procedure used for measuring the erythromycin N-demethylase activity was identical with that used for N, N-dimethylaniline demethylase activity as same standard curve was used. All the experiment was run in duplicates and repeated. The average values were considered.

4.14. Enzyme kinetics

Michaelis-Menten constant (Km) and maximal velocity (Vmax) were used to define the enzymatic activity. Km and Vmax values were determined by use of nonlinear regression analysis (rate of metabolite formation against substrate concentration) using solver module in Microsoft excel 2007 (Fig. 4.11).

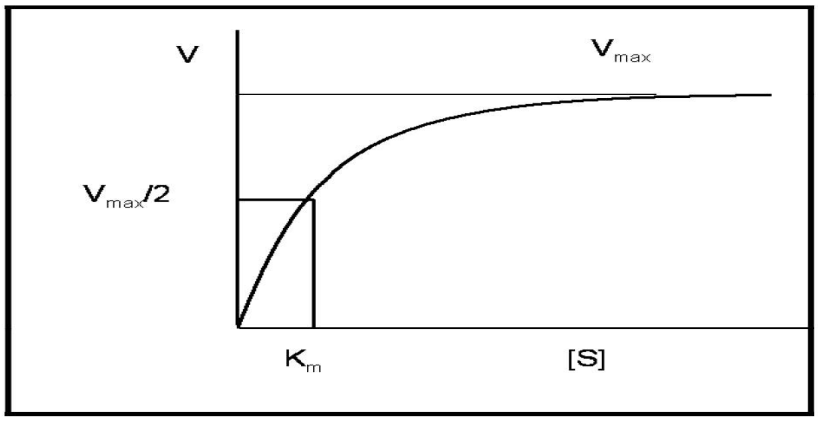

Figure 4.11. Diagram of reaction speed and Michaelis-Menten kinetics showing the increase in reaction velocity (V) with the increase in substrate concentration ([S]).

As the substrate concentration increases, saturation of substrate binding to the enzyme active site eventually occurs and a maximal reaction velocity (Vmax) is reached. The substrate concentration at a reaction velocity which is half Vmax is called the Km and is a measure of

the affinity of the enzyme for the substrate. The reaction velocity (V) at any particular substrate concentration ([S]) is given by one site binding model in our study (Fig. 4.11):

$$V = \frac{V_{max} \times [S]}{K_m + [S]}$$

In vitro kinetics of hepatic phase I biotransformation reactions in 3 air breathing teleost fish, *C. punctatus*, *H. fossilis* and *C. batrachus* were carried out. The fishes were treated with a sub-lethal concentration of the pesticide cypermethrin, ethion and dicofol for a period of 15 days and enzyme kinetics studied against the control. Maximal velocity (Vmax), binding affinity (Km) and catalytic efficiency (Vmax/Km) were used as endpoints for comparison.

4.14.1. Specific experimental parameters for each substrate

4.14.1.1. EROD

7-ethoxyresorufin concentrations ranged from 0.125 μM to 3.0 μM. Microsomal protein concentration was approximately 150 μg/ml and the reaction mixture was incubated for 10 min. The metabolite standard for 7-ethoxyresorufin metabolism was resorufin. The assay protocol is the same as mentioned in section 4.13.1.

4.14.1.2. N, N-dimethylaniline demethylation

The N, N-dimethylaniline concentration ranged from 0.025 mM to 1.2 mM. The microsomal protein concentration was approximately 1 mg/ml. Incubation times were 30 min. The formation of formaldehyde at the end of the reaction was measured for N, N-dimethylaniline metabolism. The assay protocol is the same as mentioned in section 4.13.2.

4.14.1.3. Aniline hydroxylation

Aniline concentrations ranged from 0.25 mM to 12 mM. The microsomal protein concentration was approximately 1 mg/ml and the incubation time of the reaction mixture was 30 min. Metabolite standard for aniline metabolism was p-aminophenol. The assay protocol is the same as mentioned in section 4.13.3.

4.14.1.4. Erythromycin N-demethylation

Erythromycin concentration ranged from 0.025 mM to 1.2 mM. The microsomal protein concentration was approximately 1mg/ml and the incubation time was 10 min. The formation of formaldehyde at the end of the reaction was measured for erythromycin metabolism. The assay protocol is the same as mentioned in section 4.13.4.

4.15. Gel electrophoresis

Reagents

- **30% Stock Acrylamide:** 29.2 gm acrylamide and 0.8 gm bisacrylamide were dissolved in 100 ml distilled water.
- **1.5 M Tris buffer:** 18.171 gm Tris was dissolved in 80 ml distilled water. The pH was adjusted to 8.8 using HCl and the final volume was maintained at 100 ml.
- **0.5 M Tris buffer:** 6.057 gm Tris was dissolved in 80 ml of distilled water. The pH was adjusted to 6.8 using HCl and the final volume was maintained at 100 ml.
- **20% SDS (Sodium dodecyl sulfate):** 2 gm SDS was dissolved in 10 ml distilled water.
- **10% APS (Ammonium persulfate):** 0.1 gm APS was dissolved in 1 ml distilled water.
- **Sample buffer for SDS-PAGE (reducing- 5 ml):** 0.625 ml Tris pH-6.8, 0.5 ml β-mercaptoethanol, 1 ml glycerol and 1 ml 10% SDS was dissolved in 1.875 ml distilled water.
- **Electrode buffer pH 8.3:** 1.5 gm Tris, 7.15 gm glycine and 1 gm SDS was dissolved in 500 ml distilled water.
- **Bromophenol blue (0.05%):** 0.05 gm bromophenol blue was dissolved in 100 ml distilled water.
- **0.25% Coomassie Blue staining solution:** 0.25 gm Coomassie Blue was dissolved in a solution containing 50 ml methanol, 10 ml acetic acid and 40 ml distilled water.
- **Destaining solution:** 50 ml methanol, 10 ml Acetic acid and 40 ml distilled water was mixed together to make a solution of 100 ml.
- **TEMED**
- **β-Mercaptoethanol**

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of microsomes prepared from the liver of different fish was done by the method of Laemmli (1970). SDS-PAGE was performed in a vertical electrophoresis unit using a 10% separating gel and 5% stacking gel with a discontinuous tris-glycine buffer system for differentiating the CYP 450 bands. Bromophenol blue was used as tracking dye. The gel was stained by 0.25% coomassie blue stain for overnight and destained the following day to visualize the protein band. The methods for preparing 10% separating gel and 5% stacking gel is presented in table 4.2.

Table 4.2. Reaction mixture and assay protocol for preparing 5% stacking gel and 10% resolving gel.

Reagents	**5% stacking gel**	**10% resolving gel**
30% Stock Acrylamide	1.7 ml	10.0 ml
Tris	1.8 ml (pH 6.8)	11.2 ml (pH 8.8)
SDS (Sodium dodecylsulfate)	0.10 ml	0.15 ml
Distilled water	6.5 ml	8.65 ml
TEMED	0.05 ml	0.1 ml
APS (Ammonium persulfate)	0.05 ml	0.1 ml

4.15.1. Heme staining

Reagents

- **0.05 M Tris-HCl - pH 7.0:** 0.605 gm Tris was dissolved in 80 ml distilled water. The pH was adjusted to 7.0 using HCl and the final volume was maintained at 100 ml.
- **TMPD (N,N,N'N' -tetramethyl-p-phenylenediamine)**
- **30% hydrogen peroxide**

The electrophoresis procedure was employed to visualize the heme-containing proteins chiefly CYP 450. PAGE gels were prepared as described earlier except that SDS was omitted from the gels (Thomas *et al.*, 1976). Prior to loading, the gels were prepared and pre-run for

20 min at 180 V to remove excess ammonium persulfate and to allow SDS from the running buffer to enter the gels before protein was loaded. Microsomal protein was diluted with sample buffer and incubated for 5 min at 70°C before being loaded into individual wells. As β-mercaptoethanol inhibits the peroxidase staining reaction, it was omitted from the sample buffer (Welton and Aust, 1974). Gels were run at a constant 120 V till the tracking dye reached the end of the gel. Gels were then placed into 50 ml of Tris–HCl (0.05 M, pH 7.0) containing 15 mg TMPD (N,N,N'N' -tetramethyl-p-phenylenediamine) and 75 μl of 30% hydrogen peroxide to visualize hemeprotein bands.

4.16. Isolation of genomic DNA

Reagents

- **Proteinase K (10 mg/ml Stock solution):** 10 mg proteinase K was dissolved in 1 ml of distilled water.
- **SET buffer pH-8:** 0.3 M Sucrose (10.26 gm in 100 ml), 0.5 M EDTA (18.612 gm EDTA in 100 ml) and 1 M Tris pH-8 (12.114 gm Tris in 100 ml) were mixed in equal volume.
- **Lysis buffer (TEN):** 1 M Tris pH-8 (12.114 gm Tris in 100 ml), 0.5 M EDTA (18.612 gm EDTA in 100 ml) and 5 M NaCl (29.22 gm NaCl in 100 ml) were mixed in equal volume.
- **Tris/EDTA buffer (TE) pH-8:** 1 M Tris pH-8 (12.114 gm Tris in 100 ml) and 0.5 M EDTA (18.612 gm EDTA in 100 ml) were mixed in equal volume.
- **Chloroform/ isoamyl alcohol (24:1):** 24 ml chloroform and 1 ml isoamyl alcohol was mixed together.
- **5 M NaCl:** 29.22 gm NaCl was dissolved in 100 ml distilled water.

Genomic DNA was isolated from 1 gm of the liver sample by phenol chloroform isoamyl method (Sambrook and Russel, 2001). The schematic DNA extraction method is presented in Figure 4.12. DNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. The relative purity was evaluated by computing the ratio of A260 to A280 where a ratio of 1.8-2.0 was considered highly pure. An O.D value of 1 corresponds to 50 μg of double stranded DNA. The DNA was electrophoresed on 1.0% agarose gel and visualized after staining with 0.2% ethidium bromide.

DNA extraction protocol

1. The tissue was rinsed and homogenized in cold SET buffer followed by proteinase K (0.1 mg/ml) digestion at 4°C for 15 minutes. The solution was then centrifuged at 5000 rpm X 5 minutes.
2. An aliquot of the pellet (200-500 µl) was taken. The volume was made up to 900 µl with TEN buffer and 100 µl of 10% SDS was added and incubated at 50°C for 10 minutes.
3. The solution was then cooled to room temperature and 250 µl 5M NaCl was added and kept on ice for 30 minutes after gentle vortexing. The solution was then centrifuged at 10000 rpm X 15 minutes.
4. The clear volume was transferred to a fresh centrifuge tube. An equal volume of saturated phenol was added and centrifuged at 8000 rpm X 10 minutes at 4°C.
5. The clear volume was transferred to a fresh centrifuge tube. An equal volume comprising of saturated phenol + chloroform: isoamyl alcohol (24:1) was added and centrifuged at 8000 rpm X 10 minutes at 4°C.
6. The clear volume was transferred to a clean centrifuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) solution was added and centrifuged in 8000 rpm X 10 minutes at 4°C.
7. The clear volume was transferred into a clean centrifuge tube and double volume of chilled absolute ethanol was added and stored at -20°C for 30 minutes. The solution was centrifuged at 13000 rpm X 5 minutes at 4°C.
8. The ethanol was flushed from the tubes and a good volume of 70% ethanol (cold) was added and centrifuged at 13000 rpm X 5 minutes at 4°C.
9. The ethanol was flushed and the tube was dried at 50°C until no trace of ethanol or moisture was detected.
10. The extract was then dissolved in 50 µl TE Buffer and stored at 4°C overnight to assure dissolving to be homogenous.
11. Finally, the extract was transferred to -20°C for storage and future use.

Figure 4.12. Schematic representation for isolation of DNA from liver tissue.

4.17. Amplification of CYP1A isoform from genomic DNA using PCR

Reagents

- **25 µl Master Mix for PCR amplification:**

Reaction buffer	-	2.5 µl
dNTP	-	0.5 µl
primer	-	2 µl
Double distilled water	-	17.75 µl
DNA polymerase	-	0.25 µl
Template	-	2 µl

The CYP1A isoform from all the three fish species, *Channa punctatus*, *Heteropneustes fossilis* and *Clarias batrachus* was amplified from isolated genomic DNA by PCR using the primers presented in table 4.3 and sent to SciGenome Lab for sequencing.

The PCR program or cycle conditions for *Channa punctatus*:

- *Initial denaturation for 5 min at 94°C,*
  35 cycles
- *Denaturation for 45 sec at 94°C,*
- *Annealing for 1min at 65°C,*
- *Extension for 1 min 30 sec at 72°C.*
- *Final extension 8 min at 72°C.*

The PCR program or cycle conditions for *Heteropneustes fossilis* and *Clarias batrachus* :

- *Initial denaturation for 5 min at 94°C,*
  35 cycles
- *Denaturation for 25 sec at 98°C,*
- *Annealing for 45 sec at 58°C,*
- *Extension for 1 min 30 sec at 72°C.*
- *Final extension 8 min at 72°C*

Table 4.3. Oligonucleotide primers used in PCR amplification of genomic CYP1A

Channa punctatus-

Sense (F)1	5'- ACA ACG GTG TGT CTG GTC TAC -3'
Antisense (R) 1	5'- GAC AAT GCA GTG GTG ACA GTG -3'
Sense (F) 2	5'- CTG CCG ACT TCA TCC CCA TT -3'
Antisense (R) 2	5'- TGA ACC TTA CAG TTC CTG AGT GA -3'
Sense (F) 3	5'- AGA TGT TGC GGC ACT CTT CA -3'
Antisense (R) 3	5'- TCT GCT CCG TAC AGA AAC CTC -3'

Heteropneustes fossilis and *Clarias batrachus*-

Sense (F)	5'- CGA GGG TGA GAG TTC TGA GT -3'
Antisense (R)	5'- CAG CTT CCT GTC CTC ACA GT -3'

4.18. Phylogenetic analysis

DNA sequences with the following Genbank accession numbers were retrieved from the database and used in the phylogenetic analysis along with the genomic DNA sequences of *Channa punctatus, Heteropneustes fossilis* and *Clarias batrachus* which has been submitted by us in the Genbank : JX480500.2 (*Catla catla* CYP1A), AB048939.1 (*Cyprinus carpio* CYP1A), AF015660.1 (rainbow trout CYP1A), EU683728.1 (*Channa maculate* CYP1A), U14162.1 (scup CYP1A), FJ389918.2 (*Nile tilapia* CYP1A), EF584508.1 (*Peltobagrus fulvidraco* CYP1A), HM043796.1 (*Ancistrus sp*. CYP1A).

In order to determine homology among CYP1A family cDNAs or deduced amino acid sequences from various species, sequence alignment was performed by the CLUSTAL W method using Mega6 (Ver. 6.06).

4.19. Statistical analysis

All the data are presented as mean ± standard deviation (SD). Statistical analysis was carried out using t-test and one-way analysis of variance (ANOVA). The means were compared using Dunnett's test and Tukey HSD test. Km and Vmax values were determined by using nonlinear regression analysis (rate of metabolite formation against substrate concentration) using solver module in Microsoft excel 2007. The statistical significance was tested at 0.1, 1 and 5 % levels. All the analysis were performed using SPSS version 16.0. The documentation of SDS-PAGE and TMPD stained gels was done using Spectronics ImageAide software, version 3.06.04 and phylogenetic analysis with sequence alignment was performed by the CLUSTAL W method using Mega6 (Ver. 6.06).