9. APPENDICES

Appendix A

List of published research papers in Journals:

- **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2015.** Hepatic Cytochrome P450 as Biomarkers of Cypermethrin Toxicity in Freshwater Teleost, *Channa punctatus* (Bloch). *Brazilian Archives of Biology and Technology*.58: 130-135. (ISSN 1516-8913).

- **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2013.** Detection of Multiple Cytochrome P450 in Hepatic Tissue of *Heteropneustes fossilis* (Bloch) Exposed to Cypermethrin. *Proceedings of the Zoological Society*. 66: 14–19. (ISSN 0373-5893).

- **Dawa Bhutia, Benoy K. Rai and J. Pal. 2010**. Multiple forms of cytochrome P450 family in liver of fresh water teleost fish, *Heteropneustes fossilis (Bloch)*. *N.B.U. Journal of Animal Sciences*. 4: 28-35. (ISSN 0975-1424).

List of submitted genomic DNA sequences (CYP1A) in the GenBank:

Channa punctata isolate CHANNA cytochrome p450 CYP1A (cyp1A) gene, partial cds
GenBank: KP282054.1---- **Dawa Bhutia,** Benoy K. Rai and Joydeb Pal. 2015

Channa punctata isolate NBUC1 cytochrome p450 CYP1A (cyp1A) gene, partial cds
GenBank: KP203843.1---- **Dawa Bhutia,** Benoy K. Rai and Joydeb Pal. 2015

Channa punctata isolate NBUC2 cytochrome p450 CYP1A (cyp1A) gene, partial cds
GenBank: KP231221.1---- **Dawa Bhutia,** Benoy K. Rai and Joydeb Pal. 2015

Channa punctata isolate NBUC3 cytochrome p450 CYP1A (cyp1A) gene, partial cds
GenBank: KP271996.1---- **Dawa Bhutia,** Benoy K. Rai and Joydeb Pal. 2015

Heteropneustes fossilis partial cyp4501a gene for Cytochrome P450 1A
GenBank: LN736019.1---- **Dawa Bhutia,** Benoy K. Rai and Joydeb Pal. 2015

Clarias batrachus isolate HM514 cytochrome p450 CYP1A (cyp1A) gene, partial cds
GenBank: KP336485.1---- **Dawa Bhutia,** Benoy K. Rai and Joydeb Pal. 2015

Appendix B

List of abstracts published in proceedings of Seminars/ Conferences/ Workshops

1. **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2010.** Cytochrome P450 induction in liver microsomes of air breathing teleost fish, *Channa punctatus*, *Heteropneustes fossilis* and *Clarias batrachus* after exposure to pyrethroid insecticide, Cypermethrin. National Symposium on "*Pesticide stress on Target, Non-Target Organisms and Human Health*". Department of Zoology, University of North Bengal (oral presentation).
2. **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2010.** "Studies on some Cytochrome P450 family in freshwater teleost fish, *Heteropneustes fossilis* and *Labeo rohita"*. National Conference on *"Evaluation of Biodiversity of Eastern Himalaya and Adjoining Plains"*. Department of Zoology, University of North Bengal in collaboration with Centre for Environment and Development, Kolkata (oral presentation).
3. Participated in the "**UGC Sponsored Research Scholars' Training Programme**", from 30th June 2011 to 1st July 2011 at UGC Academic Staff College, University of North Bengal.
4. **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2012.** "Expression of hepatic Cytochrome P450 in *Heteropneustes fossilis* (Bloch) upon exposure to Cypermethrin". National Conference on *"Research in Animal Science: Development and Evaluation"*. Department of Zoology, University of North Bengal (oral presentation).
5. **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2013.** Expression of multiple forms of cytochrome P450 family in liver of freshwater teleost fish, *Heteropneustes fossilis* (bloch). National Conference on *"Man, Animal and Environment interaction in the perspective of modern Research"*. Department of Zoology, University of North Bengal (oral presentation).
6. **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2014.** "Effects of cypermethrin on the activities of hepatic cytochrome P450 in *Channa punctatus* (Bloch)", UGC sponsored National seminar on *"Biological Research in Human Welfare"*. Department of Zoology, University of North Bengal (poster presentation).
7. **Dawa Bhutia, Benoy K. Rai, Joydeb Pal. 2015.** Hepatic Cytochrome P450 in Freshwater Teleost, *Channa punctatus* (Bloch) as Biomarkers of Cypermethrin Toxicity. National Conference on "*Applied Zoology in Sustainable Development: An Update*". Department of Zoology, University of North Bengal (oral presentation).

Appendix C

REPRINTS OF PUBLICATIONS

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A N I N T E R N A T I O N A L J O U R N A L

Hepatic Cytochrome P450 as Biomarkers of Cypermethrin Toxicity in Freshwater Teleost, *Channa punctatus* (Bloch)

Dawa Bhutia, Benoy Kishore Rai and Joydeb Pal*
Ecology and Toxicology Laboratory; Department of Zoology; University of North Bengal; Siliguri, West Bengal - India

ABSTRACT

In this study, Channa punctatus *was treated with sub-lethal concentration of cypermethrin (6.6 µg/L) for 5, 10 and 15 days and its effect on total CYP 450 and the activity of hepatic CYP450 isoforms measured. Total CYP450 content and CYP1A mediated EROD activity was significantly induced ($p<0.05$) in all three treated groups compared to control whereas only 15 days treated group showed significant induction in CYP2B mediated N,N-dimethylaniline demethylase activity. CYP2E1 mediated aniline hydroxylase activity showed only a marginal increase while there was inhibition of CYP3A4 mediated erythromycin demethylase activity. Liver somatic index (LSI) also showed a marginal increase in all the treated groups. Results showed differential induction of CYP1A, CYP2B, CYP2E1 and inhibition of CYP3A4 isoform due to cypermethrin treatment in* C. punctatus**.** *The study clearly showed CYP1A isoform as the most responsive and important biomarker for monitoring the aquatic pollution.*

Key words: cypermethrin, cytochrome P450, fish, *Channa punctatus*, biomarker

INTRODUCTION

Pesticide usage is a critical concern which may have an adverse effect on the delicate ecosystem because of their toxicity, persistency and tendency to concentrate in organisms as they move up in the food chain, increase their toxicity to fish, birds and other wildlife and, in turn to man. While the pesticides are instrumental in achieving significant increase in crop productivity, they also cause serious ecological hazards to the non-target animals especially the fish, which forms an important part of food chain for various animals including human beings (Sharma and Ansari 2011).

Synthetic pyrethroids have been introduced over the past two decades for agricultural and domestic use as replacements for more toxic pesticides, such as chlorinated hydrocarbons, organophosphates and carbamates. Cypermethrin is a type of cyanophenoxybenzyl pyrethroid and is categorized as restricted use pesticide by US EPA because of its high toxicity to fish (Saha and Kaviraj 2009).

Fish, as a bioindicator species, plays an increasingly important role in the monitoring of water pollution because it responds with great sensitivity to the changes in the aquatic environment. Biochemical markers are measurable responses to the exposure of an organism to xenobiotics. Regarding biochemical responses in fish to aquatic pollutants, cytochrome P450 (CYP 450) is known to play a major role in the oxidative metabolism/biotransformation of a wide range of both endogenous and exogenous compounds and is considered one of the most

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important phase I biotransformation enzymes (Siroka and Drastichova 2004). The families responsible for the metabolism of xenobiotics are CYP1 to CYP3, and to a much lesser extent CYP4, whereas cytochrome P450 enzymes belonging to other families are concerned with the metabolism of endogenous substrates (Ioannides and Lewis 2004).

CYP 450 is very sensitive to a wide range of xenobiotics and is usually the first detectable and quantifiable response to environmental change, including a change in the chemical environment. Understanding of toxicological mechanisms have provided an advancement in biological tools for environmental assessments and CYP 450 family is widely studied as a biomarker for environmental contamination in aquatic ecosystems. These tools, called biomarkers, are biological responses at molecular, cellular, or organism levels and have accordingly been classified as biomarkers of exposure, effect, and susceptibility (Moore et al. 2004).

The effects of cypermethrin on commercially important Indian species of fish are poorly documented. The objective of the present study was to evaluate if activities of hepatic cytochrome P450 could be evaluated as potential biomarkers of cypermethrin toxicity to fish.

MATERIALS AND METHODS

Fish

Specimens of *Channa punctatus* were collected from the local fish market. Fish were acclimatized in the laboratory for a period of two weeks. After that, healthy fish (male and female) weighing approximately 35± 5 g were transferred to glass aquarium with 50 L capacity in controlled light (12 hr light/12 hr dark) and aeration conditions. They were fed regularly with small pieces of chopped fish at fixed rate during the course of the experiment.

Experimental design

Fish were randomly taken in six groups (eight fish in each aquarium) each of control and treated. A sub-lethal concentration of 6.6 µg/L cypermethrin based on acute toxicity data (not published) generated from the laboratory experiments was used to treat the experimental fish. The water was renewed every 48 h with fresh pesticide for the treated groups as cypermethrin has been reported to be inactive after 72 h (Saha and Kaviraj 2008) and only water for the control group. Homogeneity was maintained in all the groups by providing similar experimental conditions. At the end of the experiment, livers were excised, weighed and the liver somatic index (LSI) was determined as percentage ratio of liver weight to body weight. The liver somatic index was calculated by using the formula: LSI = (liver weight/total live weight) X 100. As the liver samples were too small to be processed individually for enzyme activity, they were pooled (eight fish livers each) before homogenization.

Microsome isolation

Microsomes were isolated using the procedure described by Chang and Waxman (1998). 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 in four volumes of homogenization buffer (1.15% KCl, 1 mM EDTA and 0.05 M Tris, pH 7.4) using a teflon homogenizer. The homogenate was centrifuged in a cooling centrifuge at 12000 g for 20 min. The supernatant was subjected to centrifugation at 100000 g for 60 min at 4ºC in a super speed vacuum centrifuge. The resultant pellet were resuspended in two volumes of resuspension buffer containing 0.05 M Tris, 1mM EDTA and 20% Glycerol v/v, pH 7.4 as the hepatic microsomal fraction.

Enzyme assay

Sectral analysis of CYP 450 was based on the mepthod described by Omura and Sato (1964). Protein in the microsomal fraction was estimated as described by Lowry et al. (1951) using bovine serum albumin as standard. Ethoxyresorufin O-deethylase (EROD) activity in the liver microsome samples was determined spectrophotometrically by the method of Klotz et al. (1984).

NNDMA activity was determined by the method of Schenkman et al. (1967) with minor modifications to detect CYP2B activity. Reaction mixture consisted of 100 mM N, N-dimethylaniline, 150 mM $MgCl_2$, 100 mM semicarbazide and microsomes 1.5-2.0 mg. The mixture was pre-incubated for 5 min at 32ºC and the reaction started by adding 10mM NADPH. Following aerobic incubation for another 30 min the reaction was terminated by

adding 0.5 mL each of 25% zinc sulfate and saturated barium hydroxide. Formaldehyde formed during the assay was measured by the method of Nash (1953) at 412 nm.

Aniline hydroxylase activity (CYP2E1) was determined by measuring the amount of p-aminophenol formed at 630 nm. The method was modified from Imai et al. (1966) by using aniline (10 mM) as substrate and NADPH (10 mM) instead of an NADPH generating system. In addition, the incubation mixture consisted 100 mM $MgCl_2$, 120 mM tris (pH 7.4) and 1.5-2.0 mg microsomal protein. After pre-incubation for 5 min at 32ºC, the reaction was initiated by the addition of NADPH and incubated for 30 min.

Erythromycin demethylase (CYP3A4) activity was measured following the method of Werringloer (1978) at 412 nm. The reaction was incubated at 32ºC for 10 min. Formaldehyde formed as the end product was measured by the method of Nash (1953) at 412 nm. All the assays were done in duplicate.

Statistical analysis

Data were expressed as mean ± standard deviation and were analyzed using one-way analysis of variance (ANOVA) followed by Dunnet's test. The statistical significance was tested at 5% levels.

RESULTS

Total CYP 450 was significantly induced in all the three treated groups (5, 10 and 15 days) with higher induction seen in the groups with increasing number of days when compared to control (Table 1). The carbon monoxide difference spectra of dithionite reduced liver microsomes of *C. punctatus* were studied at 1 min interval over a period of 5 min (Fig. 1). The maximum absorbance was observed at 1 and 2 min and then gradual decrease in absorbance was seen with increase in time interval.

The LSI values were higher in all the treated groups (Table 1). EROD (CYP1A) activity was significantly induced in all the three treated groups with highest induction shown by the 15 days treated group as expected. The activity increased with the exposure time (Fig. 2).

Five and 10 days treated groups showed no significant increase in N, N-dimethylaniline (N, N-DMA) demethylase (CYP2B) activity. The activity was significantly induced only in the 15 days treated group when compared to the control (Fig. 3).

No significant increase in aniline hydroxylase (CYP2E1) activity was observed in 15 days treated group whereas 5 and 10 days treated groups showed no induction (Fig. 4).

Table 1 - Total CYP 450 content and liver somatic index (LSI) of *Channa punctatus.*

Parameter	control	5 days	10 days	15 days	F-value	p-value
Total CYP450 content (nmol/mg protein)	0.276±0.089	0.359±0.020*	0.382±0.042*	0.425 ±.053*	6.190	0.003
Liver Somatic Index [LSI %]	0.914±0.113	0.985±0.096	1.009±0.120	1.042±.109	1.924	0.155

values are Mean ± SD (n=6). * significantly different from control

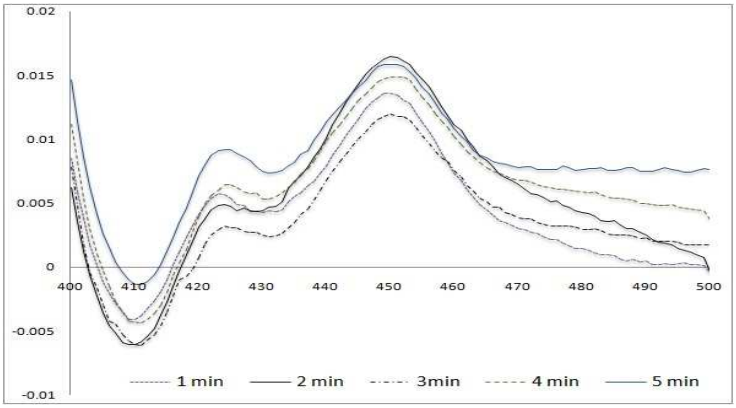

Figure 1 - Carbon monoxide difference spectra of dithionite reduced liver microsomes of *Channa punctatus* at 1 min interval over a period of 5 min.

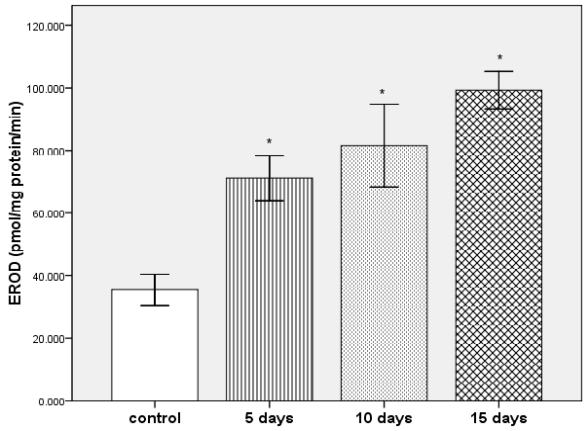

Figure 2 - CYP1A mediated EROD activity in liver of *Channa punctatus.* * indicates significant difference from control ($p<0.05$).

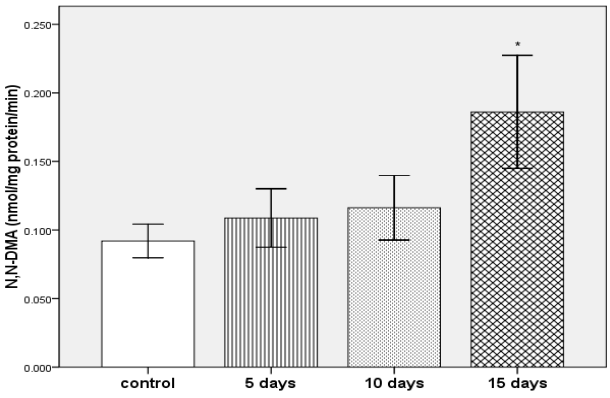

Figure 3 - CYP2B mediated N, N-dimethylaniline demethylase (N, N-DMA) activity in liver of *Channa punctatus.* *indicates significant difference from control ($p<0.05$).

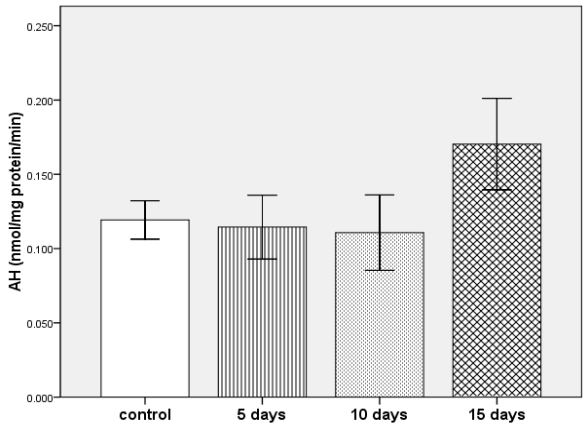

Figure 4 - CYP2E1 mediated aniline hydroxylase (AH) activity in liver of *Channa punctatus.*

All the three treated groups showed an inhibition in Erythromycin demethylase (CYP3A4) activity (Fig. 5). But only 10 and 15 days treated groups resulted in significant difference with respect to the control. The percentage of inhibition is shown in parenthesis.

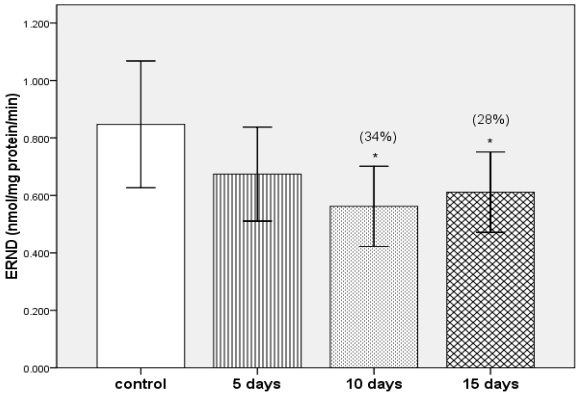

Figure 5 - CYP3A4 mediated erythromycin demethylase (ERND) activity in liver of *Channa punctatus.* *indicates significant difference from control ($p<0.05$).

DISCUSSION

Hepatic CYP 450 expression of fish can be induced by exposure to xenobiotics because specific CYP1A expression is highest in this organ. Most lipophilic chemicals, including drugs, pesticides, carcinogens, environmental pollutants and naturally occurring compounds undergo enzyme-mediated oxidative, hydrolytic or conjugative biotransformation in liver and in extra hepatic tissues, yielding more polar metabolites that can be easily excreted. Thus, metabolism plays a critical role in determining both the efficacy and the residence time of drugs in the body as well as in modulating the response to toxic chemicals (Akdogan and Sen 2010).

Two soret peaks were seen in the dithionite reduced spectra of liver microsomes (Fig. 1). These peaks consisted of the characteristic absorbance at 450 nm for the reduced CYP 450-CO complex and the other of lesser magnitude at around 420-425 nm, which could be possibly due to the absorbance of contaminating hemoglobin since tissues could not be adequately perfused (Klemz et al. 2010).

Liver somatic index (LSI) has frequently been used as a biomarker for examining fish exposed to contaminants. LSI values are generally elevated in

the vertebrates experiencing induction of hepatic microsomal P450 for the detoxification of organic compounds and LSI is generally used to determine the physiological status of the fish (Shailaja et al. 2006). Increase in LSI is commonly seen in fish exposed for long periods of time to organic contaminants in the laboratory and field. The increase in LSI can be due to hyperplasia (increased cell number) and/or hypertrophy (increased cell size). It may be associated with increased capacity to metabolize xenobiotics so it could be considered as adaption to the presence of pollution rather than a dysfunction (Heath 1995).

Enzyme activity increases with increase in exposure period to the xenobiotics as it brings about the physiological differences within the organism (Arellano-Aguilar et al. 2009). Each isoform of the cytochrome P450 participates in the metabolism of many different compounds, but one substrate can also be metabolized by several different isoforms (Siroka and Drastichova 2004). 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 activity of EROD. In this study, the EROD activity was induced in all the treated groups, probably due to EROD`s higher detection sensitivity. Well-established inducers of CYP1A and EROD are organic contaminants belonging to PCBs, PAHs, PCDDs and PCDFs. However, induction by pesticide compounds has also been reported (Haluzova et al. 2011).

Bhutia et al. (2010) reported the absence of CYP2B in fish, *H. fossilis* as it did not respond to the mammalian specific CYP2B inducer, Phenobarbital. However, Stegeman et al. (1997) reported the presence of CYP2B like activities in four tropical fish species using catalytic activity assay and detection of the CYP2B protein by an immunoblot assay. Similarly, Bhutia et al. (2013) also reported the induction of CYP2B like activities in fish, *H. fossilis* after exposure to pesticide, cypermethrin. It is likely that some P450s differ in their functions among the mammals and fish and follow different mechanisms of induction. In consequence, whenever P450 enzymes are used as biomarkers in monitoring programs, one should be cautious with metabolic differences, even among fish species.

CYP2E1 mediated p-hydroxylation has been shown to be a possible pathway for the metabolism of xenobiotics in the liver of fish species. This activity can be detected by studying *in vitro* transformation of toxic aniline to nontoxic derivative *p*-aminophenol (Zen and Korkmaz 2009). Although, CYP2E1 is responsible for the metabolism and potential bioactivation of a number of low-molecular-weight pharmaceutical and other xenobiotic compounds, including acetaminophen, ethanol, isoniazid, halogenated anesthetics, acetone, and benzene (Omiecinski et al. 1999), a study by Bhutia et al. (2013) has reported its role in metabolism of pesticide with long-term exposure.

This study showed the inhibition of CYP3A4 activity which was somewhat surprising given that CYP3A is one of the most abundant P450 isoform in fish liver possessing a broad range of substrate specificity (Hegelund and Celander 2003) and accounts for the metabolism of almost 50% of the currently used pharmaceutical agents (Parkinson 1996). This could also be the reason for high toxicity of pyrethroid insecticide in fish species as CYP3A4 was reported to be induced in *Labeo rohita* (Rai et al. 2010) and channel catfish (Stuchal et al. 2006) after exposure to chlorpyriphos and methoxychlor.

The present study clearly demonstrated the usefulness of CYP 450 as an important biomarker for monitoring the aquatic pollution. Of all the CYP 450 isoforms (CYP1A, CYP2B, CYP2E1 and CYP3A4), CYP1A was the most sensitive isoform towards cypermethrin toxicity and in accordance to various other studies as one of the most established biomarker of CYP 450 family.

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Proc Zool Soc (Jan-June 2013) 66(1):14–19
DOI 10.1007/s12595-013-0063-6

RESEARCH ARTICLE

Detection of Multiple Cytochrome P450 in Hepatic Tissue of *Heteropneustes fossilis* (Bloch) Exposed to Cypermethrin

Dawa Bhutia · Benoy K. Rai · Joydeb Pal

Received: 2 July 2012 / Revised: 13 February 2013 / Accepted: 17 March 2013 / Published online: 28 March 2013

Abstract Cypermethrin (alpha-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylic acid) is a synthetic pyrethroid. It is one of the most widely used pesticide in commercial agricultural applications because of its high effectiveness against target species. Beside its target toxicity it is also highly toxic to other non-target species like fish, bees and aquatic insects. The aim of this study was to detect the presence of cytochrome P450 (CYP 450) in the hepatic microsomes of *Heteropneustes fossilis* upon exposure to cypermethrin. The 96 h LC_{50} value for each exposure route was calculated and two groups were treated, with one group receiving a single IP (intraperitoneal) injection for 96 h (0.030 mg/kg body weight) and the other group with 1/3 sub-lethal concentration (1.2 μg/l) of the LC_{50} value in water for 15 days. Activities of the enzymes ethoxyresorufin-*o*-deethylase (EROD), *N,N*-dimethylaniline demethylase, aniline hydroxylase and erythromycin demethylase mediated respectively by the isozymes CYP1A, CYP2B, CYP2E1 and CYP3A4 were studied. The liver somatic index (LSI) was also calculated to determine the physiological status of the fish. Activities of CYP1A, CYP2B and CYP2E1 enzymes increased significantly while that of CYP3A4 enzyme inhibited as compared to control. Total CYP 450 content was also significantly induced in both the treated groups. The increase in activities of CYP P450 isozymes could be used as a biomarker to indicate the pollution of an aquatic environment by the pesticide.

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Keywords Pesticide · Fish · Biomarker · EROD · Aquatic pollution

Introduction

Contamination of the environment with anthropogenic compounds is a widespread phenomenon and occurs both from fixed and diffused source. Pesticides carried away by rains and floods to larger water bodies like ponds and rivers alter the physico-chemical properties of water. Cypermethrin, the alpha-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylic acid is a widely used pesticide based on pyrethroids and account for almost over 30 % of the global pesticide use (Dahamna et al. 2011). Although synthetic pyrethroids are less persistent and less toxic to mammals and birds, they are highly toxic to a number of non-target organisms such as bees, freshwater fish and other aquatic organisms even at a very low concentration (Oudou et al. 2004).

Biomarkers so called biological responses to xenobiotics have been studied as environmental pollution indicators and used as early warning systems of environmental contamination (Walker et al. 1996). The biochemical marker that is best studied so far is the stimulation of cytochrome P450-dependent monooxygenases (CYP 450) which is also considered the most dominant enzyme system responsible for oxidation processes in phase I biotransformation. Payne and Penrose (1975) were among the first to make use of this enzyme system as a biomarker, reporting elevated CYP 450 activity in fish from petroleum-contaminated sites in the aquatic environment and members of CYP 450 gene families 1–4 are considered prominent in xenobiotic metabolism. Thus, CYP 450 enzymes are important in the mechanisms underlying chemically induced toxicity or

disease and chemical–ecological interactions. Fish species are useful as bioindicators, since they are promptly exposed to diverse anthropogenic water contaminants and in the fish, CYP 450 has primarily been studied as a biomarker indicating pollution of the aquatic environment by industrial or agricultural sewage. However, responses to xenobiotics in fish may differ from those in other species (Siroka and Drastichova 2004).

This work was aimed to evaluate the effects of cypermethrin on CYP1A, CYP2B, CYP2E1and CYP3A4 enzymes as biomarkers in *Heteropneustes fossilis*. Standardization of biomarkers for detection of sub-lethal effects of cypermethrin in this species under laboratory conditions will enable biomonitoring of cypermethrin in the environment.

Materials and Methods

Heteropneustes fossilis were collected from non-contaminated local ponds of Gossaipur of Darjeeling district. Fishes were acclimatized in the laboratory for a period of 2 weeks. After 2 weeks, healthy fish (male and female) weighing approximately 35 ± 5 g were transferred to glass aquarium with 50 l capacity in controlled light (12 h light/12 h dark), and aeration conditions. The fishes were fed regularly with small pieces of chopped fish during acclimatization and feeding stopped during the course of treatment.

The 96 h LC_{50} value was calculated using probit analysis (Finney 1971). Fish were randomly taken in 5 groups (10 fishes in each aquarium of 20 l capacity). The 96 h LC_{50} value for *H. fossilis* was determined as 3.783 µg/l (data not published). One group of the experimental fish (5–7 fish) was treated with sub lethal concentration (1.2 µg/l) of cypermethrin, Ripcord 10 % EC (1/3 of LC_{50} value) for 15 days. The water was renewed every 2 days and the desired concentration of the pesticide was maintained before introducing the treated group while only water was changed for the control groups.

Similarly, another group was administered with a single IP (intraperitoneal) injection of 0.03 mg/kg body weight of the pesticide. The 96 h IP LC_{50} value was determined as 0.090 mg/kg body weight (data not published). The pesticide was diluted with distilled water and the positive (+VE) control fishes received only an IP injection of distilled water in the same volume as the injected group. Homogeneity was maintained in all the groups by providing similar experimental conditions. Two treated and the +VE control groups were studied for the experiment to determine the stress level, specificity and interaction of the pesticide with respect to the control.

The fish were sacrificed by a single blow on the head and then livers were excised, weighed and the liver somatic index (LSI) was determined as percentage ratio of liver weight to body weight. The liver somatic index was calculated by using the formula: LSI = (liver weight/total live weight) × 100. As the liver samples were too small to be processed individually for enzyme activity, they were pooled before homogenization.

Microsomes were isolated using the procedure described by Chang and Waxman (1998), with minor modifications. Livers were perfused with a large volume of ice cold perfusion buffer (1.15 % KCl, 1 mM EDTA, pH 7.4) to get rid of unwanted tissues, fat bodies and blood and homogenized in four volumes of homogenization buffer (1.15 % KCl, 1 mM EDTA and 0.05 M Tris, pH 7.4) using a Teflon homogenizer. The homogenate was centrifuged in a cooling centrifuge at 12,000×*g* for 20 min. The supernatant was ultra centrifuged at 100,000×*g* for 60 min at 4 °C in a super speed vacuum centrifuge. The pellet were resuspended in two volumes of resuspension buffer containing 0.05 M Tris, 1 mM EDTA and 20 % Glycerol v/v, pH 7.4 to obtain the hepatic microsomal fraction.

Analysis of CYP 450 was based on the method described by Omura and Sato (1964) with minor modifications. The microsomal preparation was diluted in 0.1 M phosphate buffer and saturated with carbon monoxide for 40 s. The sample was equally divided between two cuvettes. The baseline (400–500 nm) was recorded and a few milligrams of sodium dithionite were added to only one of the cuvette in order to reduce CYP450 and the absorbance measured in a spectrophotometer.

Protein in the microsomal fraction was estimated as described by Lowry et al. (1951). The values of protein were determined from the standard curve prepared from OD values against different concentrations of bovine serum albumen standard.

EROD (ethoxyresorufin *O*-deethylase) was determined by spectrophotometric method of Klotz et al. (1984). The reaction mixture consisted of (0.1 M) NaCl, (2 µM) 7-ethoxresorufin, (0.1 M) tris buffer-pH 7.8 and 100–200 µg of microsomes. After pre-incubation at 30 °C for 5 min the reaction was initiated by the addition of (0.01 M) NADPH and the reaction was stopped following 10 min of incubation at the same temperature with 0.5 ml of ice cold trichloroacetic acid. After incubation, the amount of resorufin formed was measured at 572 nm.

Procedure of Schenkman et al. (1967) was adopted with minor modifications to determine the *N,N*-dimethylaniline (*N,N*-DMA) demethylase and aniline hydroxylase activity. The reaction mixture for *N,N*-DMA activity consisted of the (0.1 M) phosphate buffer-pH 7.4, (0.1 M) *N,N*-dimethylaniline, (0.15 M) $MgCl_2$, (0.1 M) semicarbazide and microsomes (1.0 mg). The mixture were incubated for

five minutes at 32 °C and the reaction started by adding (0.1 M) NADPH instead of NADPH generating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $MgCl_2$). Following aerobic incubation for another 30 min 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. Formaldehyde formed as the end product of *N,N*-dimethylaniline demethylase activity was measured by the method of Nash (1953) at 412 nm.

The reaction mixture for aniline hydroxylase activity consisted of (0.1 M) aniline, (0.1 M) $MgCl_2$, (0.12 M) tris-pH 7.4 and 1.0 mg of microsomal protein. The reaction mixture temperature was raised to 32 °C by incubation in a water bath for 5 min and the reaction was started by adding (0.1 M) NADPH. The reaction was terminated after 30 min by adding 0.5 ml of ice cold 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 *p*-aminophenol formed as the end product of aniline hydroxylase activity was measured at 630 nm.

Erythromycin demethylase activity was measured following the method of Werringloer (1978) at 412 nm. The reaction mixture consisted of (0.05 M) phosphate buffer-pH 7.25, (0.01 M) erythromycin, (0.15 M) $MgCl_2$ and microsomes (1 mg). Reactions were initiated by the addition of (0.1 M) NADPH, and the mixture was incubated for 10 min at 32 °C. The amount of formaldehyde formed was measured follwing the method of Werringloer (1978) at 412 nm. The reaction was terminated by adding 0.5 ml of 20 % trichloroacetic acid. All assays were done in duplicate and repeated twice.

Differences between groups of animals or treatments were analysed using one-way analysis of variance (ANOVA) followed by Least Significant difference (LSD) test. The statistical significance was tested at 1 and 5 % levels.

Results and Discussion

Both the treated groups showed significant stimulation ($p < 0.05$) with respect to the control in the total CYP450 content (Table 1). The 15 days sub-lethal treated group showed higher stimulation (0.523 nmol/mg protein) than the 96 h IP treated group (0.509 nmol/mg protein). The +VE control group did not show any significant difference with the control group. The increase in CYP 450 content after the treatment positively correlated with the increase in the enzymatic activity.

Both the treated groups and the +VE control group showed marginal increase in LSI with respect to that of the control but the means were not statistically significant. When all the groups were considered, the 96 h IP treated group showed higher LSI value than the 15 days sub-lethal treated and the control groups (Table 1).

Table 2 shows the enzymatic activity reflecting stimulation of CYP1A, CYP2B and CYP2E1 and inhibition of CYP3A4 isozymes. The treated groups, i.e. 96 h IP treated and the 15 days sub-lethal treated showed similar trend in the enzymatic activities when compared with the control in all the 4 enzyme activities studied—EROD, Aniline hydroxylase, *N,N*-dimethylalinile demethylase and Erythromycin demethylase. Similar results were also displayed by the control and the +VE control group. No significant difference in the enzyme activities was seen between the control and the +VE control (injected with distilled water alone) and also between 15 days sub-lethal and 96 h IP treated groups following injection with cypermethrin.

EROD activity was significantly stimulated ($p < 0.01$) by both the treatment regimes of cypermethrin. The 15 days treated group showed higher stimulation than the 96 h IP treated group. An increase of almost 1-fold in the activity was seen in both the treated group compared to the control and the +VE control (Table 2). Both the treated group showed stimulation in *N,N*-dimethylaniline demethylase activity compared to the control. The 15 days treated group of *H. fossilis* showed the highest stimulation with an increase of 225 % while 167 % increase was seen in 96 h treated group (Table 2). Aniline hydroxylase

Table 1 Total CYP 450 content and liver somatic index (LSI) of *H. fossilis*

Parameter	Control ($n = 7$)	+ve Control ($n = 5$)	96 h IP treated ($n = 5$)	15 days sub-lethal treated ($n = 6$)	*F*-value	*p* value
CYP 450 content (nmol/mg protein)	0.313 ± 0.061	0.294 ± 0.133^{ns}	0.509 ± 0.069*	0.523 ± 0.234*	4.233	.019
Liver somatic index [LSI %]	0.988 ± 0.140	1.108 ± 0.098^{ns}	1.192 ± 0.296^{ns}	1.029 ± 0.227^{ns}	1.140	.358

Values are Mean ± SD

+*v* positive, *ns* not significant

* Significantly different ($p \leq 0.05$)

Table 2 EROD, *N,N*-dimethylaniline (*N,N*-DMA) demethylase, aniline hydroxylase (AH) and erythromycin demethylase (ERND) activity of hepatic microsomes in *H. fossilis*

Parameter	Control ($n = 7$)	+ve control ($n = 5$)	96 h IP treated ($n = 5$)	15 days sub-lethal treated ($n = 6$)	F value	p value
EROD[a]	34.450 ± 3.357	33.886 ± 3.592[ns]	61.897 ± 9.968**	63.661 ± 10.766**	20.412	0.000
N,N-DMA[b]	0.112 ± 0.064	0.154 ± 0.115[ns]	0.300 ± 0.058*	0.364 ± 0.164**	7.634	0.002
AH[b]	0.104 ± 0.094	0.169 ± 0.061[ns]	0.375 ± 0.163*	0.249 ± 0.045*	7.185	0.002
ERND[b]	0.743 ± 0.168	0.770 ± 0.120[ns]	0.504 ± 0.166*	0.505 ± 0.142*	4.398	0.021

Values are Mean ± SD

* Significantly different ($p \leq 0.05$), ** ($p \leq 0.01$), p value indicates significant difference between the control and different treated groups

+*ve* positive, *n* not significant with the control

[a] Expressed as (pmol/mg protein/min)

[b] Expressed as (nmol/mg protein/min)

activity was significantly stimulated ($p < 0.05$) in both the treated group. The 96 h IP treated group of *H. fossilis* showed the highest stimulation with an increase of 260 % than that of 15 days treated group with 139 % when compared with the control. No stimulation in the CYP3A4 was shown by both the treated groups of fish studied. Also there was no significant difference in the activities of CYP 450 isozymes between the control and the +VE control.

The carbon monoxide difference spectra of dithionite reduced liver microsomes of *H. fossilis* were studied at 1 min interval over a period of 5 min. The maximum absorbance was noticed at 1–2 min and then a gradual decrease in absorbance was seen with increase in time interval. This time interval corresponds to the length of incubation required for maximum reduction of the enzyme by sodium dithionite. The CYP 450 converts to a stable denatured state CYP 420 under long periods of incubation thus adding to the absorbance at 420 nm. Two soret peaks were seen in the dithionite reduced spectra of liver microsomes (Fig. 1). These peaks consisted of the characteristic absorbance at 450 nm for the reduced CYP450-CO complex and the other of lesser magnitude at around 420–425 nm which may be possibly due to the absorbance of contaminating hemoglobin or CYP 420 (Klemz et al. 2010).

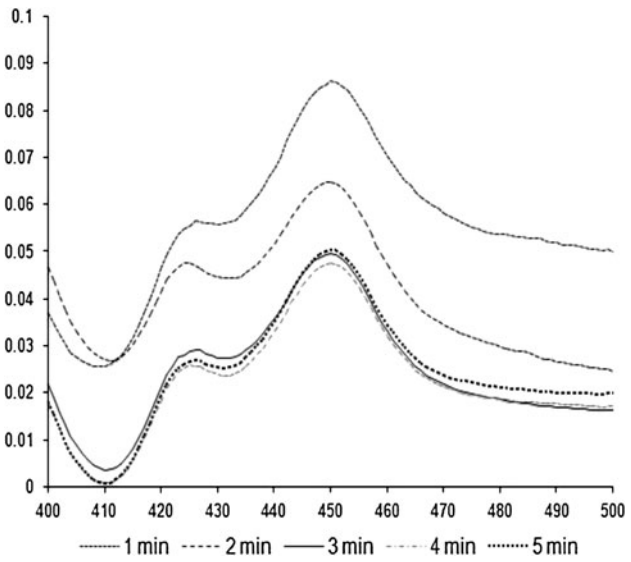

Fig. 1 Carbon monoxide difference spectra of dithionite reduced liver microsomes of *H. fossilis* at 1 min interval over a period of 5 min

LSI has frequently been used as a biomarker for examining fish exposed to environmental contaminants and also to determine the physiological status of the fish. LSI values are generally elevated in vertebrates experiencing stimulation of hepatic microsomal CYP 450 and the elevation in LSI may be due to the altered allocation of energy reserves for detoxification of organic compounds (Miller et al. 2009).

The biochemical responses assessed in the present study are the earliest indicators of exposure to stressors that can be detected in an organism. CYP 450 metabolizes and biotransforms most lipophilic xenobiotics including drugs, pesticides, carcinogens and environmental pollutants into more polar metabolites that can be easily excreted from the body and are useful as early warning indices of environmental alteration, so that remedial actions may be taken by resource protection managers in time to prevent permanent decline in fish populations (Thomas 1990).

It has been detected that variability of enzyme activity is higher if the organism in test is exposed for longer duration to the xenobiotic or pollutants as it brings about the physiological differences within the organism (Arellano-Aguilar et al. 2009). This may be the reason that the activities of CYP450 is slightly higher in 15 days sub-lethal treated compared to the 96 h IP treated group. Although an IP injection of test agents is not an environmentally relevant route of exposure, however, for pharmacological considerations, it is a common practice of exposure to chemical in animals as it allows a direct effect on the target organs (Rice and Roszell 1998). Fish can also

be subjected to desired concentrations of pesticides through injection to cut down the time of exposure and also for understanding the effects on biotransformation enzymes (Assis et al. 2009).

CYP 450s, principally the CYP1A isoform have been used to detect the presence of pollutants in aquatic environment due to its high affinity to xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) and have been so far proved to be the most sensitive indicators (Schlenk and Di Giulio 2002). 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 been conducted that establishes CYP1A stimulation in response to pesticides. Lemaire et al. (2010) have reported induced level of EROD activity in liver microsomes of roundnose grenadier in response to DDT exposure. Similar results have been confirmed in fishes exposed to deltamethrin (Assis et al. 2009) and chlorpyriphos (Rai et al. 2010).

Earlier studies have reported that Phenobarbital, specific inducer of CYP2B in mammals have not been found to induce CYP2B in fish (Bhutia et al. 2010), although CYP2B like activities do occur in fish as reported by Stegeman et al.(1997). It may be possible that pesticides do induce CYP2B like activities (Price et al. 2008) in fish. Though specific CYPs are necessary to metabolize xenobiotics, pesticides have the ability to induce one or more forms of CYP 450 simultaneously.

Aniline hydroxylation by cytochrome P450-dependent enzyme systems has been shown to be a possible way for metabolism in the liver of fish species. Aniline hydroxylase activity is suggested to be mainly catalyzed by enzymes belonging to the CYP450 2E1 sub-family in fish as in mammals and studies have reported an stimulation of CYP2E1 activity upon exposure to pesticides carbaryl (Tang et al. 2002) and parathion (Mutch et al. 2003).

Both the treated groups showed significant inhibition in erythromycin demethylase activity although CYP3A is reported to be induced in *Labeo rohita* exposed to chlorpyriphos (Rai et al. 2010). It is seen that CYP3A4 is the predominant isoform responsible for metabolizing pyrethroid pesticides in mammals (Scollon et al. 2009), thus, a potential explanation for the high pyrethroid toxicity in fish can be due to the inhibition of CYP3A4 isoform in the liver and other tissues.

The present study demonstrates evidence of CYP 450 related xenobiotic metabolism in the liver of *H. fossilis* exposed to cypermethrin. The CYP 450 mediated catalytic activity may also be a very efficient adaptive strategy of the species to increase tolerance to the pesticides and guarantee its survival. Although field trials were not done, based on the laboratory results it was concluded that at least the CYP450 mediated enzyme activities in *H. fossilis* could serve as a useful biomarker of cypermethrin pollution in the aquatic environment.

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MULTIPLE FORMS OF CYTOCHROME P450 FAMILY IN LIVER OF FRESHWATER TELEOST FISH, *Heteropneustes fossilis* (Bloch)

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ABSTRACT

Cytochrome P450 (CYP450) is a very large and diverse super family of heme proteins found in all domains of life. Among the diverse family of Cytochrome P450, family 1, 2, and 3 are of prime importance for the oxidation of xenobiotic compounds. Except for cytochrome P450 1A (CYP1A) information on other CYP450 families in fish is limited as compared to mammals. In the present study, CYP1A, CYP2E1, CYP2B and CYP3A-like activity have been reported from liver microsomes of fish, *Heteropneustes fossilis*. CYP1A-dependent EROD activity, CYP2B-dependent N, N-dimethylaniline demethylase activity, CYP3A-dependent erythromycin demethylase activity and CYP2E1-dependent aniline hydroxylase activity were detected in liver microsomes. Induction of specific enzyme activities upon treatment with inducers such as naphthalene, phenobarbitone, deflazacort and acetone, specific for the CYP450 family was found except for CYP2B.

Key words: Cytochrome P450, *Heteropneustes fossilis*, CYP1A, CYP2E1, CYP2B, CYP3A.

INTRODUCTION

Cytochrome P450 is a very large and diverse super family of heme proteins found in bacteria, archaea and eukaryotes. The term cytochrome P450 refers to a family of heme proteins present in all mammalian cell types, except mature red blood cells and skeletal muscle which catalyzes oxidation of a wide variety of structurally diverse compounds, such as steroids and fatty acids (including prostaglandins and leukotriens) and exogenous compounds, such as drugs, food additives or industrial by-products that enter the body through the skin (Okita and Masters, 1997). Several hundred CYP450s have been characterized in different species of organisms that have been studied (Nelson *et al*., 1996). A standardized nomenclature has been adopted based primarily on sequence similarities to categorize them into various families and subfamilies. P450 proteins exhibiting 40% similarity in amino acid sequence are classified within the same family and exhibiting 55% sequence similarity are grouped into the same subfamily (Nebert and McKinnon, 1994; Nelson *et al.*, 1996).

Cytochrome P450, the most important group of phase I biotransformation enzymes, are involved in detoxification and elimination of foreign compounds from the body (Anzenbacher and Anzenbacherová, 2001). Fish plays an important role in monitoring of water pollution because it responds with great sensitivity to changes in aquatic environment (Saha and Kaviraj, 2003). The induction of cytochrome P450 isoforms can be used as biochemical markers of pollution in fish exposed to toxic compounds in the aquatic system. Biochemical markers are measurable responses to the exposure of an organism to xenobiotics. In fish the cytochrome P450 has primarily being studied as biomarker indicating pollution of the aquatic environment by industrial or agricultural sewage. Cytochrome P450 responds to

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water contamination when it is too low or when the contaminant is no longer dissolved in water but persist only in residues of living matter (Machala *et al*., 1997).

In fish, cytochrome P450 1A (CYP1A) activities are often used as a marker to determine the quantities of persistent organic pollutants (Havelkova *et al.,* 2007). Induction of cytochrome P450 1A in fish has been widely used as a sensitive and convenient early warning signal of exposure to aryl hydrocarbon agonists such as polychlorinated biphenyls, dioxins, furans, organochlorine pesticides and polyaromatic hydrocarbons (Payne *et al.*,1987; Goksoyr and Forlin, 1992; Beyer *et al.*, 1996; Jonsson, 2003). In mammals, phenobarbital (PB) is an *in vivo* inducer of the cytochrome P450 2B (CYP2B) family, whereas in teleosts PB induction of cytochrome P450 is unclear (Sadars *et al.*, 1996). Cytochrome P450 3A (CYP3A) plays an important role in the metabolism of endogenous substances and xenobiotics including pharmaceuticals. Cytochrome P450 2E1 (CYP2E1) metabolises many endogenous or exogenous small molecules such as acetone, glycerol, ethanol, acetaminophen, carbon tetrachloride, halothane or nitrosamines (Gonzalez, 2005).

In the present study the fish, *Heteropneustes fossilis* were treated with specific inducers to study whether fish cytochrome P450 shows similar activities like that of their mammalian counterpart.

MATERIALS AND METHODS

Collection and maintenance of healthy fish

Healthy *Heteropneustes fossilis* weighing an average of 35±2 gm were collected from commercial ponds of Sonapur of Darjeeling district. 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 was maintained at 20cm. The fishes were fed regularly with chopped fishes. The fishes were acclimatized for at least fifteen days under laboratory conditions before the start of experiment.

Dose treatment

Fishes were given a single intraperitoneal injectin of 20 mg/kg bodyweight of naphthalene, phenobarbitone and deflazacort and 2 ml/kg body weight of acetone. After an exposure of 72 hours, the enzyme activities were studied with respect to control groups.

Preparation of Microsomes

The procedure for preparation of microsomes was based on the methods reported by Nilsen *et al*. (1998), Chang and Waxman (1998), with minor modifications. A sample of liver tissues (0.5 to 2.0g) were placed in ice cold homogenization buffer containing 1.15% KCl, 1mM EDTA and 0.05M Tris, pH 7.4 (4 ml per gram of tissue) and homogenized. The homogenized tissues were centrifuged in a cooling centrifuge (REMI C 24) at 12,000xg for 20 min at a temperature of 4^{o}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 1,00,000xg for 60 minutes at 4^{o}C. The supernatant were discarded (cytosol fraction) and the microsomal pellet were resuspended in a resuspension buffer containing 0.05M Tris, 1mM EDTA and 20% Glycerol, pH 7.4 (2 ml per gram of original tissue weight). The fraction were maintained on ice for immediate analysis or stored at -20^{o}C for future use.

Protein Estimation

Protein in the microsomal fraction was estimated as described by Lowry *et al.* (1951) using bovine serum albumin as standard protein.

Spectral analysis of cytochrome P-450

Spectral analysis of cytochrome P450 in the hepatic microsomal fraction was done using the method described by Omura and Sato (1964). Briefly diluted microsome preparation was mixed with a few milligrams of sodium dithionite in order to reduce CYP450. The sample was equally divided between two cuvettes. The baseline (400nm to 500nm) was

recorded following which only one of the cuvette was saturated with carbon monoxide for 30 seconds 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.

Determination of N, N-dimethylaniline demethylase activity

Demethylation assay was carried out by the procedure of Schenkman *et al*. (1967). Formaldehyde formed during the assay was measured by the method of Nash (1953). Microsomal suspension (0.5 ml), was mixed with a reaction mixture consisting of the substrate 0.1 M (N, N-dimethyl aniline), 0.15M $MgCl_2$, 0.1 M semicarbazide. After pre-incubation at 30±2 °C for five min the reaction was started by adding 40µl NADPH and incubated for 30 minutes at the same temperature. The reaction was stopped by the addition of 0.5 ml of each of 25% zinc sulfate and saturated barium hydroxide followed by centrifugation at 10,000xg for 10 mins. Aliquot of clear supernatant (1ml) was mixed with 2ml of Nash reagent and incubated at 60 °C for 30 minutes and the absorbance measured at 415nm. The experiment was run in duplicates and repeated. The average values were considered.

Determination of aniline hydroxylase activity

Aniline hydroxylase assay was carried out by the procedure of Schenkman *et al*. (1967). The reaction mixture consisted of 0.5 ml microsomal suspension, 0.1 M aniline, 0.1M $MgCl_2$, 0.12 M tris (pH 7.4) and preincubated at 30±2 °C for 5 min. The reaction was started by adding 0.01 M NADPH following incubation at the same temperature. The reaction was terminated by adding 0.5 ml of 20% trichloroacetic acid(TCA). Precipitated proteins were removed by centrifugation at 10,000g for 10 min and 1 ml of supernatant were added to 1 ml of a solution containing 2% phenol in 0.2 M NaOH and 1 ml of Na_2CO_3. After 30 min incubation, the resultant blue colour of p-aminophenol formed was measured at 630 nm.

Determination of erythromycin demethylase activity

Erythromycin demethylase assay was carried out by the procedure of Werringloer (1978). Formaldehyde formed during the assay was measured by the method of Nash (1953). The reaction mixture consisted of 0.1 M potassium phosphate buffer ((pH 7.2), 0.01 M erythromycin, 0.15 M $MgCl_2$. Reactions were initiated by the addition of 0.01 M NADPH, and the mixture was incubated for 10 min at 30 ± 2 °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 and 1 ml of supernatant were added to 1 ml of Nash reagent and measured at 412 nm.

Determination of EROD (ethoxyresorufin O-deethylase) activity

EROD (ethoxyresorufin O-deethylase) assay was carried out by the procedure of Klotz *et al*. (1984). The reaction mixture consisted of 0.1 M NaCl, 2 µM 7-ethoxresorufin, 0.1 M tris buffer (pH 7.4). The reaction was initiated by the addition of 0.01 M NADPH. After incubation at 30 ± 2 °C for 10 min the reaction was terminated by the addition of 20% trichloroacetic acid and centrifuged at 20,000 g for 10 min and the activity measured at 572 nm.

Statistical analysis

Data was analyzed (Students *t*- test, paired two sample for means) using Microsoft Analysis ToolPak.

RESULTS AND DISCUSSION

Lester *et al*. (1993) and Lewis (2001) reported high levels of CYP 450 in the fish liver, accounting for 1 to 2% mass of hepatocytes. A difference spectrum with a peak absorbance at 450 was observed upon dithionite reduction followed by bubbling of CO, showing the presence of cytochrome P450 in the microsomal fraction. The highest content of total CYP 450, 0.440 nmole/mg protein was recorded with deflazacort treated fish compared to

0.214 nmole/mg protein in control group. Overall the fish treated with different inducers showed a higher content of CYP 450 with respect to control (Table I). Figure 1 highlights the peak absorbance with the control showing a maximum peak at 450 nm (0.01208), naphthalene at 451 nm (450 = 0.03127; 451 = 0.03129), phenobarbitone at 452 nm (450 = 0.01884; 451 = 0.01929), acetone at 450 nm (0.02629) and that of deflazacort treated at 449 nm (450 = 0.03091; 449=0.03093). The determination of cytochrome P450 levels as a response of the organism to the presence of pollutants in the aquatic environment has been reported by many studies from all over the world (Payne *et al.*, 1987; Curtis *et al.*, 1993; Aas *et al.*, 2001). An increase in the level of CYP 450 was reported in hepatic microsomal fraction of *Heteropneustes fossilis* when treated with

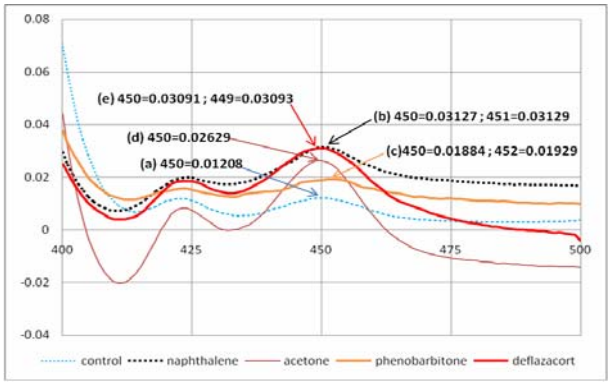

Figure 1. Carbon monoxide difference spectra of dithionite reduced liver microsomes of *Heteropneustes fossilis* treated with different inducers, (a) control, (b) naphthalene treated, (c) phenobarbitone treated, (d) acetone treated and (e) deflazacort treated.

Table I. Total cytochrome P450 content administered with different inducers with respect to control. Values are mean of (n) number of experiments ±SD.

INDUCER/dose	CYTOCHROME P450 CONTENT (nmole/mg protein)	Absorption maxima
CONTROL VEHICLE (Water/Sesame oil)	0.214 ±0.102 (n=3)	450 nm
NAPTHELENE (20 mg/kg body weight)	0.330 ±0.089 (n=4)	451 nm
PHENOBARBITAL (20 mg/kg body weight)	0.378±0.083 (n=3)	452 nm
ACETONE (2 ml/kg body weight)	0.428 ±0.011 (n=3)	450 nm
DEFLAZACORT (20 mg/kg body weight)	0.440 ±0.096 (n=3)	449 nm

pyrethroid insecticide, cypermethrin (Sadat *et al.*, 2009).

The fish treated with different inducers showed an increase in EROD activity than that of control. EROD activity was seen to be significantly induced, 66.274 ± 23.059 ($p \leq 0.05$) only in naphthalene treated fish (Table 2). Pacheco and Santos (2002) reported that naphthalene, a class of PAH, have been found to be specific for the induction of CYP1A in European eel (*Anguilla anguilla* L.). The induction of CYP1A is mediated by the Ah receptor (AhR), a xenobiotic-binding protein present in the cytosol (Lewis, 2001). The most important inducers of fish CYP1A, which is considered most relevant to pollution of the aquatic environment, include PAHs, nitrated polyaromatic hydrocarbons (NPAHs), PCBs, dioxins and some pesticides (Machala *et al.*, 1997; Jung *et al.*, 2001).

In this study, acetone treated fish showed significant induction in aniline hydroxylase activity, 0.277±0.008 ($p \leq 0.01$) as compared to control and the fish treated with different inducers but the fish treated with naphthalene, phenobarbitone and deflazacort showed down regulation in aniline hydroxylase activity as compared to control fish (Table II). CYP2E1 is responsible for the metabolism and potential bioactivation of a number of low-molecular-weight pharmaceutical and other xenobiotic compounds, including acetaminophen, ethanol, isoniazid, halogenated anesthetics, acetone, and benzene (Lee *et al.*, 1996). Chlorzoxazone and p-nitrophenol are routinely used as substrate probes to measure CYP2E1 activity, although chlorzoxazone can also be metabolized by CYP1A2. The enzyme is inducible by ethanol, acetone, isoniazid, and by starvation in animal models (Lieber, 1997). Acetone at large dose induces CYP2E1 in rat (Tu *et al.*, 1983). The results indicate that acetone in fish, *Heteropneustes fossilis* inducing CYP2E1 as in the case of rats.

There were no significant difference in N, N-dimethylaniline (DMA) demethylase activity between the control and the treated groups (Table II). Over the last decades, fish liver microsomes have been shown to metabolize prototypical mammalian CYP2B substrates, including aldrin, benzphetamine, ethylmorphine, aminopyrine and alkoxyresorufins (Elskus and Stegeman, 1989; Haasch *et al*., 1994).

Table II. EROD, Aniline hydroxylase, N, N-dimethylaniline (DMA) demethylase and erythromycin demethylase activities treated with different inducers. Values are the mean of (n) number of experiments ± SD.

ENZYME ACTIVITY / INDUCER/dose	EROD ACTIVITY (CYP 1A) (pmole/mg protein/minute)	ANILINE HYDROXYLASE ACTIVITY (CYP2E) (nmole/mg protein/minute)	N,N-DMA DEMETHYLASE ACTIVITY (CYP2B) (nmole/mg protein/minute)	ERYTHROMYCIN DEMETHYLASE ACTIVITY (CYP3A) (nmole/mg protein/minute)
CONTROL VEHICLE (Water/Sesame oil)	17.989±12.034 (n=3)	0.037±0.028 (n=3)	0.054±0.045 (n=3)	0.898±0.062 (n=3)
NAPTHELENE (20 mg/kg body weight)	66.274±23.059* (n=4)	0.020±0.005 (n=4)	0.052±0.048 (n=3)	0.815±0.714 (n=4)
PHENOBARBITONE (20 mg/kg body weight)	41.053 ± 9.506 (n=3)	0.015±0.002 (n=3)	0.060±0.013 (n=3)	0.506±0.103* (n=3)
ACETONE (2 ml/kg body weight)	58.591± 23.376 (n=3)	0.277±0.008** (n=3)	0.036±0.005 (n=3)	0.127±0.22** (n=3)
DEFLAZACORT (20 mg/kg body weight)	38.982 ± 7.446 (n=3)	0.053±0.012 (n=3)	0.059±0.004 (n=3)	3.129±0.545** (n=3)

* Significantly different from control (*t*-test, $p \leq 0.05$), **($p \leq 0.01$).

CYP2B1 and CYP2B2 are the primary members expressed in rats. In rodents, enzymes from this subfamily are typically inducible by phenobarbital and other barbiturates, and are inhibited by metyrapone (Mimura *et al.*, 1993). In fish, however, an apparent lack of response to PB-type inducers has been observed (Haasch *et al*., 1994; Stegeman, 1981).

Deflazacort (synthetic corticosteroid) treated fish showed significant induction of erythromycin demethylase activity of 3.129±0.545 ($p \leq 0.01$) where as the other treated groups showed lower activity than that of the control group (Table II). Induction of cytochrome P450 3A expression by dexamethasone has been shown to occur in fish (Tseng *et al.*, 2005). The study also showed a significant negative regulation of erythromycin demethylase activity in acetone and phenobarbitone treated group. Bork *et al*. (1989) reported that CYP3A enzymes of mammals and fish exhibit similar catalytic properties due to structural similarities. In fish they are regulated by the same receptors as in mammals, namely, through the pregnane X receptor (Bresolin *et al*., 2005).

The results from the current study showed that fish CYP 450 responds differentially to different xenobiotics and indicated presence of multiple forms of CYP 450 in fish, *Heteropneustes fossilis*. The CYP 450 family- CYP1A, CYP2E and CYP3A show similar biotransformation reaction as that reported for various mammalian species but CYP2B did not follow the trend though still a lot of studies have to be conducted.

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Channa punctata isolate CHANNA cytochrome p450 CYP1A (cyp1A) gene, partial cds

GenBank: KP282054.1

FASTA Graphics

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1501 gtttttcttt tctcagcacc acaaaagaca cgtctctgaa tggctacttc attccaaaag
1561 atacctgtgt cttcatcaat cagtggcaga ttaaccatga tccgtaagtt ctttttgtta
1621 cattttaaaa actttaaaac aaagcatgag gtgtaccaac ctaacagtta cacagtaaaa
1681 aaactgaagc agaaaactac ttacataaaa ccattaaagg aataagtaca gaaataagtt
1741 tgtcctaatg gaatttggag ctaaagccaa agatttattg tgcttttgta tatgacttac
1801 atttcctttc tctacccctc tcagtgagct gtggaaagat ccattttcct tcaacccaga
1861 ccgcttcttg agcgctgata gcactgaggt caacaaggtg gaaggggaga aggtagtggc
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1981 cttcttggca attcttatcc agaagctaga gttccaccaa atgcctgggg taccactgga
2041 catgacgcca caatatggtc tcacaatgaa acacaaaccc tgccacctga gagccacaat
2101 gcgagcaatc aatgagcagt gaaactattt atatatttac tgtattcaga atgaattact
2161 caacagttga taattgttca ctcaagaact gtaaggttca gtgaaacaag catctttctc
2221 agaatgtacg gcaatggttg ccaaatctga tacatagagc aaatggattt gaagcaaata
2281 ggtgaaattt gcttgcttgc ttgcagactg tcagatattt ctgggtttgt aagatgaggg
2341 gttcctctat atgatcgatg cgtcttgagg aagaataagc agatacttgg ttttcctgct
2401 gtgtttttt gctgtgctgg aaacgtaact gttcttatgt agaagttgta tagcacacaa
2461 actatgttgc ttcaatcaac tttgggacac attatgtttt actggatatg c
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Channa punctata isolate NBUC1 cytochrome p450 CYP1A (cyp1A) gene, partial cds

GenBank: KP203843.1

FASTA Graphics

Go to:

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LOCUS       KP203843            1041 bp    DNA     linear   VRT 04-APR-2015
DEFINITION  Channa punctata isolate NBUC1 cytochrome p450 CYP1A (cyp1A)
            gene, partial cds.
ACCESSION   KP203843
VERSION     KP203843.1
KEYWORDS    .
SOURCE      Channa punctata (spotted snakehead)
  ORGANISM  Channa punctata
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi; Actinopterygii; Neopterygii; Teleostei;
            Neoteleostei;Acanthomorphata; Anabantaria; Anabantiformes;
            Channoidei;Channidae; Channa.
REFERENCE   1 (bases 1 to 1041)
  AUTHORS   Bhutia,D., Rai,B.K. and Pal,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2014) Department of Zoology, University of
            North Bengal, Raja Rammohanpur, Siliguri, West Bengal 734013,
            India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /isolate="NBUC1"
                     /db_xref="taxon:304456"
                     /tissue_type="liver"
                     /country="India"
                     /collection_date="04-Jul-2014"
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     mRNA            join(<1..720,845..>971)
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                     /product="cytochrome p450 CYP1A"
     CDS             join(<1..720,845..>971)
                     /gene="cyp1A"
                     /codon_start=1
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/product="cytochrome p450 CYP1A"
/protein_id="AKA59068.1"

/translation="GLCRLPGPKPLPLIGNVLELRHKPYQSLTAMSKRYGHVFQIHIG
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AYSALRSFSNLESKNSEYSCVLEEHVSKEAEYLIKRLCTVMKADGSFDPVRHIVVSVA
NVICGICFGRRYSHDDQELLSLVTLADDFNQVAGSGNPADFIPILQYLPSRNMKNFMD
LNARFNSFVQKIVREHYATYDKDNIRDITDSLIDHCEDRKLDENFNVQVSDEKIVGIV
NDLFGA"

ORIGIN

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        1 gggctttgtc gtctccctgg cccaaagccc ttgcctctca tcgggaatgt gctggagctt
       61 cgccacaaac cttatcagag tctcactgct atgagcaagc gttatggtca tgtcttccag
      121 atccacattg gcacacgtcc tgtggttgtg ttgagtggca gtgagacggt tcgtcaggct
      181 ctcatcaagc aaggggaaga gttcgcaggc agacctgact tgtacagctt tcaattcatc
      241 aatgacggaa aaagtctggc tttcagtaca gatcagtctg gtgtctggcg tgctcgcaga
      301 aagctggctt acagtgccct gcgctccttt tccaacctgg agagcaagaa ctcagagtac
      361 tcctgtgttc tagaagaaca cgtcagtaaa gaggcagagt atctaatcaa acgactctgc
      421 actgtcatga aggcagacgg cagcttcgac cccgtccgtc acattgtcgt ctctgtggca
      481 aatgtcatct gcggaatttg ctttggccga cgctacagcc acgatgacca ggagctgctc
      541 agcttagtga cccttgctga tgactttaac caggtggcgg gaagtgggaa ccctgctgac
      601 ttcatcccca ttctccagta tctgcctagc agaaacatga agaattttat ggacctcaat
      661 gctcgcttca acagctttgt gcaaaaaata gtcagagaac actatgccac ctacgacaag
      721 gtacaccgca aactaaatca ctcatgaaat gagttcacgt tttctcaaag ccataatttc
      781 cttattattt ttgtgtgtat ttgtatgtac gtatgtgatt gtgattgttc ttaacttttt
      841 tcaggacaac atccgtgata tcacagactc cctcattgat cactgcgagg acaggaagct
      901 ggatgagaac ttcaatgttc aggtgtcaga tgagaagatt gtgggaattg tcaatgacct
      961 atttggggct ggtacgctca cttcctatgt actgaataaa cctattgttg atatggtgat
     1021 gtctgcctaa agaaaacaaa a
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//

Channa punctata isolate NBUC2 cytochrome p450 CYP1A (cyp1A) gene, partial cds

GenBank: KP231221.1

FASTA Graphics

Go to:

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LOCUS       KP231221            1498 bp    DNA     linear   VRT 04-APR-2015
DEFINITION  Channa punctata isolate NBUC2 cytochrome p450 CYP1A (cyp1A)
            gene, partial cds.
ACCESSION   KP231221
VERSION     KP231221.1
KEYWORDS    .
SOURCE      Channa punctata (spotted snakehead)
  ORGANISM  Channa punctata
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi;Actinopterygii; Neopterygii; Teleostei;
            Neoteleostei;Acanthomorphata; Anabantaria; Anabantiformes;
            Channoidei;Channidae; Channa.
REFERENCE   1 (bases 1 to 1498)
  AUTHORS   Bhutia,D., Rai,B.K. and Pal,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-DEC-2014) Department of Zoology, University of
            North Bengal, Raja Rammohanpur, Siliguri, West Bengal 734013,
            India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..1498
                     /organism="Channa punctata"
                     /mol_type="genomic DNA"
                     /isolate="NBUC2"
                     /db_xref="taxon:304456"
                     /tissue_type="liver"
                     /country="India"
                     /collection_date="03-Sep-2014"
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                     /gene="cyp1A"
     mRNA            join(<1..72,197..323,426..515,622..745,868..954,
                     1176..>1473)
                     /gene="cyp1A"
                     /product="cytochrome p450 CYP1A"
     CDS             join(<1..72,197..323,426..515,622..745,868..954,
                     1176..1473)
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ENFNVQVSDEKIVGIVNDLFGAGFDTVTTALSWSVMYMVAYPEIQERLYDELKVNVGL
ERSPRLSDKPNLPFLEAFILEMLRHSSFLPFTIPHCTTKDTSLNGYFIPKDTCVFINQ
WQINHDPELWKDPFSFNPDRFLSADSTEVNKVEGEKVVAFGLGKRRCIGEVIARNEVY
LFLAILIQKLEFHQMPGVPLDMTPQYGLTMKHKPCHLRATMRAINEQ"

ORIGIN
        1 atggacctca atgctcgctt caacagcttt gtgcaaaaaa tagtcagaga acactatgcc
       61 acctacgaca aggtacaccg caaactaaat cactcatgaa atgagttcac gttttctcaa
      121 agccataatt tccttattat ttttgtgtgt atttgtatgt acgtatgtga ttgtgattgt
      181 tcttaacttt tttcaggaca acatccgtga tatcacagac tccctcattg atcactgcga
      241 ggacaggaag ctggatgaga acttcaatgt tcaggtgtca gatgagaaga ttgtgggaat
      301 tgtcaatgac ctatttgggg ctggtacgct cacttcctat gtactgaata aacctattgt
      361 tgatatggtg atgtctgcct aaagaaaaca aaagtgccct cacataatta acatctccct
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      541 cagttctatt gcaaaaccac tgaaggaatg tggactaaaa tccaaaaaaa cgcatactga
      601 gcatggatga ctttttttca gaggtcaatg tgggtctgga gcggagcccg cgtctctctg
      661 ataaacccaa tttaccattt ctggaggcct tcatcctgga gatgttgcgg cactcttcat
      721 tcctgccctt tactatccca cactggtaag gttcaactca aaaagggtga aaatgagctg
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      961 tctttttgtt acatttaaa aactttaaaa caaagcatga ggtgtaccaa cctaacagtt
     1021 acacagtaaa aaaactgaag cagaaaacta cttacataaa accattaaag gaataagtac
     1081 agaaataagt ttgtcctaat ggaatttgga gctaaagcca aagatttatt gtgcttttgt
     1141 atatgactta catttccttt ctctacccct ctcagtgagc tgtggaaaga tccattttcc
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     1381 gtaccactgg acatgacgcc acaatatggt ctcacaatga aacacaaacc ctgccacctg
     1441 agagccacaa tgcgagcaat caatgagcag tgaaactatt tatatattta ctgtatca
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Channa punctata isolate NBUC3 cytochrome p450 CYP1A (cyp1A) gene, partial cds

GenBank: KP271996.1

FASTA Graphics

Go to:

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LOCUS       KP271996            1108 bp    DNA     linear   VRT 04-APR-2015
DEFINITION  Channa punctata isolate NBUC3 cytochrome p450 CYP1A (cyp1A)
            gene,partial cds.
ACCESSION   KP271996
VERSION     KP271996.1
KEYWORDS    .
SOURCE      Channa punctata (spotted snakehead)
  ORGANISM  Channa punctata
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi;Actinopterygii; Neopterygii; Teleostei;
            Neoteleostei;Acanthomorphata; Anabantaria; Anabantiformes;
            Channoidei;Channidae; Channa.
REFERENCE   1 (bases 1 to 1108)
  AUTHORS   Bhutia,D., Rai,B.K. and Pal,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-DEC-2014) Department of Zoology, University of
            North Bengal, Raja Rammohanpur, Siliguri, West Bengal 734013,
            India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:304456"
                     /tissue_type="liver"
                     /country="India: West Bengal"
                     /collection_date="12-Nov-2014"
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                     /gene="cyp1A"
                     /codon_start=2
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TPQYGLTMKHKPCHLRATMRAINEQ"

ORIGIN

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      121 aaagacacgt ctctgaatgg ctacttcatt ccaaaagata cctgtgtctt catcaatcag
      181 tggcagatta accatgatcc gtaagttctt tttgttacat tttaaaaact ttaaaacaaa
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      301 cataaaacca ttaaaggaat aagtacagaa ataagtttgt cctaatggaa tttggagcta
      361 aagccaaaga tttattgtgc ttttgtatat gacttacatt tcctttctct acccctctca
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      481 ctgaggtcaa caaggtggaa ggggagaagg tagtggcttt cggcctagga aagcggcgct
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     1021 cgtaactgtt cttatgtaga agttgtatag cacacaaact atgttgcttc aatcaacttt
     1081 gggacacatt atgttttact ggatatgc
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//

Heteropneustes fossilis partial cyp4501a gene for Cytochrome P450 1A

GenBank: LN736019.1

FASTA Graphics

Go to:

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LOCUS       LN736019             534 bp    DNA     linear   VRT 18-JAN-2015
DEFINITION  Heteropneustes fossilis partial cyp4501a gene for Cytochrome
            P4501A.
ACCESSION   LN736019
VERSION     LN736019.1
KEYWORDS    .
SOURCE      Heteropneustes fossilis (stinging catfish)
  ORGANISM  Heteropneustes fossilis
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi; Actinopterygii; Neopterygii; Teleostei;
            Ostariophysi; Siluriformes; Heteropneustidae; Heteropneustes.
REFERENCE   1
  AUTHORS   Bhutia,D., Rai,B.K. and Pal,J.
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 534)
  AUTHORS   Bhutia,D.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2014) Ecotoxicology Laboratory, Department of
            Zoology, NBU, West Bengal, 734013, INDIA
FEATURES             Location/Qualifiers
     source          1..534
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                     /db_xref="taxon:93621"
                     /tissue_type="liver"
                     /country="India:West Bengal, Siliguri"
                     /collection_date="05-Jun-2014"
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FLAINQRFNVFMQKLVREHYETFNKDNIRDITDSLIDHCEDRKL"
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     intron          384..477
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     exon            478..>534
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      421 ctgtcaaaat tcaggatgct cacactgtct tgtatcttct gatttttgt tttttaggac
      481 aacatccgtg atatcactga ctctctcatc gatcactgtg aggacaggaa gctg
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Clarias batrachus isolate HM514 cytochrome p450 CYP1A (cyp1A) gene, partial cds

GenBank: KP336485.1

FASTA Graphics

Go to:

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LOCUS       KP336485      509 bp    DNA     linear   VRT 04-APR-2015
DEFINITION  Clarias batrachus isolate HM514 cytochrome p450 CYP1A
           (cyp1A) gene, partial cds.
ACCESSION   KP336485
VERSION     KP336485.1
KEYWORDS    .
SOURCE      Clarias batrachus (walking catfish)
  ORGANISM  Clarias batrachus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
            Euteleostomi; Actinopterygii; Neopterygii; Teleostei;
            Ostariophysi; Siluriformes; Clariidae; Clarias.
REFERENCE   1 (bases 1 to 509)
  AUTHORS   Bhutia,D., Rai,B.K. and Pal,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-2014) Department of Zoology,
            University of North Bengal, Raja Rammohanpur, Siliguri,
            West Bengal 734013, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..509
                     /organism="Clarias batrachus"
                     /mol_type="genomic DNA"
                     /isolate="HM514"
                     /db_xref="taxon:59899"
                     /tissue_type="liver"
                     /country="India: West Bengal"
                     /collection_date="16-Dec-2014"
                     /PCR_primers="fwd_name: hm-f, fwd_seq:
                     cgagggtgagagttctgagt, rev_name: hm-r, rev_seq:
                     cagcttcctgtcctcacagt"
     gene            <1..>509
                     /gene="cyp1A"
     mRNA            join(<1..366,464..>509)
                     /gene="cyp1A"
                     /product="cytochrome p450 CYP1A"
     CDS             join(<1..366,464..>509)
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                     /codon_start=1
                     /product="cytochrome p450 CYP1A"
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ICGMCFGRRYSHDDRELLSLVNLSEEFNQVVGSGNPADFIPFLRLLPSTSMKKFLAIN
ERFNVFMQRLVKEHYETYNKDNIRDITDSLIDHCE"

ORIGIN
        1 gagtactcct gcgccctgga ggaacacatc agcaaggaag gcctgtacct gattgagagg
       61 ctgcacagtg ttatgaaggc cagcggcgga ttcgacccgt tcagtcacat tgttgtgact
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