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Cytochrome P450 and its role in metabolism of dicofol in *Channa punctatus* and *Heteropneustes fossilis*

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Abstract

Pesticides and their derivatives are frequently exposed to aquatic organisms, such as fish, that live in and use the aquatic environment. Pesticide biotransformation is the first step in ensuring fish species survival. Cytochrome P450 (CYP 450) is the primary enzyme responsible for pesticide biotransformation and also serves as a biomarker for assessing aquatic environmental health. The ability of dicofol, an organochlorine pesticide, to induce CYP 450 in fish *Channa punctatus* and *Heteropnesutes fossilis* after treatment for 5, 10, and 15 days with 1/3 sub-lethal concentration (15.2 and 12.1 µg/L) of 96 hour LC_{50} value calculated in the laboratory was investigated in this study. The activities of LSI, total CYP 450 content, 7-ethoxyresorufin O-deethylase, N,N-dimethylaniline demethylse, aniline hydroxylase, and erythromycin N-demethylase were examined in liver microsomes. In comparison to their respective control, all of the enzyme activities were significantly increased in the dicofol-treated groups in both fish species. In both species, CYP1A-mediated activity was the most noticeable of all the activities. Significant induction was also seen in LSI and total CYP 450 content. The present study demonstrates that dicofol has the ability to induce CYP1A, CYP2B, CYP2E1, and CYP3A4 in the liver of fish, *Channa punctatus* and *Heteropnesutes fossilis*.

Key words : Biomarker, dicofol, *Channa punctatus*, *Heteropneustes fossilis*, cytochrome P450

Dicofol (2,2,2-Trichloro-1,1-bis(4-chlorophenyl) ethan-1-ol), an organochlorine pesticide shares chemical similarities with DDT. Dicofol is highly to extremely poisonous to all aquatic animals studied, including fish, invertebrates, and estuarine/marine organisms[41]. Since DDT serves as the raw material for the production of dicofol, it naturally contains a number of impurities[5].

A wide range of theories concerning the effects of pesticides on aquatic ecosystems,

including effects on ecosystem structure and function and its interactions with the abiotic environment, should be taken into consideration because pesticides are essential for agriculture to reduce crop damage[39]. Some pesticides, such as organochlorine insecticides, have challenges with their bioconcentrations in various fish tissues due to their low biodegradation and high lipid solubility.

Due to their keen sensitivity to changes in the aquatic environment, fish play an increasingly significant role in the monitoring of water pollution[33]. Cytochrome P450 (CYP 450) has mostly been studied in fish as a biomarker for the contamination of the aquatic environment by effluent from industry or agriculture. Biochemical markers are detectable responses to an organism's exposure to xenobiotics and are caused in the tissues by the presence of a particular class of pollutants[37]. *In vitro* and *in vivo* assays are used to measure biochemical markers in toxicology, ecotoxicology, and pharmacology research.

Although CYP 450 enzymes are found in nearly all tissues, liver endoplasmic reticulum contains the largest concentration of those involved in xenobiotic biotransformation[18]. Heme-thiolate proteins belonging to the large multigene CYP 450 family play a key role in the metabolism of xenobiotics and endobiotics, and hepatic clearance is the main method for xenobiotic removal[28]. By using various *in vitro* methods, it is now possible to distinguish between metabolic processes and interactions and to identify which CYP 450 is responsible for the metabolism of a certain xenobiotic[14,27,29].

Cytochrome P450 isoforms are of vital significance in the metabolism of many xenobiotics and endogenous compounds[45]. Due to its high sensitivity to xenobiotics like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, and heavy metals, total CYP 450 has been used to detect the presence of pollutants in aquatic environments and has thus far been proven to be the most responsive indicator[35,44].

Numerous studies on the reaction of CYP 450 to pesticides have been released. Aldicarb, a carbamate insecticide, was found to stimulate the expression of various CYP 450 isoforms in *Ictalurus punctatus*[30]. The fungicide propiconazole was also found to induce the expression of hepatic CYP1A in brown trout[10]. According to Manar *et al.*[20], 18 pesticides that are part of the pyrethroid, benzoyl urea, and organophosphorous pesticide families have been found to induce and inhibit CYP 450, primarily of the family 1-3.

The effects of dicofol are poorly documented. The goal of the current study was to assess the effects of dicofol on the hepatic CYP 450 enzyme activities in *Channa punctatus* and *Heteropneustes fossilis*, two commercially significant Indian fish species. It was also intended to assess whether CYP 450 could be used as potential biomarker of dicofol toxicity to fish.

Design of the experiment :

The fish, *Channa punctatus* and *Heteropneustes fossilis* (30±5 gm) were collected and kept in 50 L capacity glass aquarium with sufficient aeration in controlled light conditions (12 hr light/12 hr dark). After two weeks of acclimatization, the experiment

was started. The regulatory rules set by University of North Bengal Animal Ethics Committee were maintained to carry out the experimental procedure.

Four groups of fish were randomly selected (eight fish in each aquarium) for control and dicofol treated. The fish, *C. punctatus* and *H. fossilis* were exposed to 15.2 and 12.1 µg/L of dicofol (1/3 of LC_{50} value) for a period of 5, 10 and 15 days based on toxicity data (not published) generated from the laboratory experiments. The water was renewed every 48 hr with 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. The fishes were then sacrificed at the end of 5, 10 and 15 days, and livers were excised, weighed and the liver somatic index (LSI) was determined as percentage ratio of liver weight to body weight. As the liver samples were too small to be processed individually for enzyme activity, they were pooled (eight fish livers each) before homogenization.

Isolation of microsomes :

The procedure described by Chang and Waxman[7] was followed for the isolation of microsomes at 4 p C with minor modification. Initially the livers were perfused with a perfusion buffer (1.15% KCl, 1mM EDTA, pH 7.4) and then the tissues were cut into small pieces and homogenized in four volumes of homogenization buffer (1.15% KCl, 1mM EDTA and 50 mMTris, pH 7.4). The homogenate was centrifuged at 12000 g for 20 min. The supernatant was then subjected to centrifugation at 100000 g for 60 minutes. Finally the pellet was resuspended in two volumes (tissue weight) of resuspension buffer containing 50 mMTris, 1mM EDTA and 20% Glycerol v/v, pH 7.4 to obtain the hepatic microsomal fraction.

Protein content was estimated by the method of Lowry *et al*.[19] using bovine serum albumin as standard.

Enzyme assay :

Detection and estimation of CYP 450 was done following the method described by Omura and Sato[23]. The CYP 450 content was determined by using the extinction coefficient (ΔE450-490) of 91 mM^{-1} cm^{-1}.

The method of Klotz *et al*.[16] was employed to determine CYP1A specific EROD (7-Ethoxyresorufin O-deethylase) activity spectrophotometrically. The brown colour of resorufin formed at the end of the reaction was measured at 572 nm. The reaction was carried out at 32°C.

The method described by Schenkman *et al*.,[16] was employed to estimate CYP2B mediated N, N-dimethylaniline demethylase (N,N-DMA) activity and CYP2E1 mediated Aniline hydroxylase (AH) activity with minor modifications. The reaction mixture was incubated for 30 minutes at 32°C and initiated by adding (10mM) NADPH instead of NADPH-generating system. The formaldehyde formed as the end product in N,N-DMA activity was measured by the method of Nash[22] at 412 nm. The amount of p-aminophenol formed at the end of AH activity was measured at 630 nm in a spectrophotometer.

The method of Werringloer[42] was followed for the determination of CYP3A catalyzed erythromycin N-demethylase (ERND) activity. Formaldehyde formed during the assay was measured by the method of Nash[22] at 412 nm.

Statistical analysis :

Data are represented as mean ± SD and were analyzed using one-way ANOVA followed by Dunnet's test using SPSS version 16.0. The statistical significance was tested at 1 and 5% levels.

Liver somatic index and CYP 450 content :

Table-1 shows the LSI values of control and dicofol treated fish, *C. punctatus* and *H. fossilis*. All of the dicofol treated fish displayed significant difference in LSI values when compared with their respective control group. In *C. punctatus*, the mean value of LSI increased significantly in all the treated groups ($p<0.05$; $p<0.01$). In *H. fossilis*, the 10 days ($p<0.05$) and 15 days ($p<0.01$) treated group revealed a significant increase in LSI value while the 5 days treated group only showed a marginal increase in comparison to the control.

Table-2 illustrates the CYP 450 content in control and dicofol treated fish, *C. punctatus* and *H. fossilis*. In *C. punctatus*, no significant difference was seen between control and 5 days treated group while the 10 days ($p<0.05$) and 15 days ($p<0.001$) treated groups displayed a significant difference. *H. fossilis* revealed a significant difference ($p<0.001$) only in 15 days treated group in comparison to its control group. The highest induction was seen at 15 days treated group with.2.1 fold increase in *H. fossilis* and 1.8 fold increase in *C. punctatus* when compared with their respective control group.

EROD, N,N-DMA, AH and ERND activities:

The entire dicofol treated groups of two fish species varied significantly ($p<0.01$) in EROD activity compared to their respective control group (Fig. 1). In *C. punctatus*, the mean value increased after 5 days, then a drastic increase was seen at 10 days, thereafter, the activity decreased slightly after 15 days of exposure. On the other hand, in *H. fossilis* increasing trend of EROD activity was seen after 5, 10 and 15 days of exposure.

In both *C. punctatus* and *H. fossilis*, only the 15 days treated group displayed a significant induction ($p<0.01$) in comparison to the control (Fig. 2). The 5 and 10 days treated group displayed only a marginal difference in N,N-DMA activity.

In *C. punctatus*, the AH activity decreased marginally after 5, 10 and 15 days of exposure but was not significant. *H. fossilis*, on the other hand, displayed a significant increase ($p<0.01$) in all the treated groups (Fig. 3). The values reflected a 3.1, 2.6 and 3.8 fold elevation after 5, 10 and 15 days of exposure when compared to its control.

The ERND activity in *C. punctatus* showed an increasing trend with significant difference in all 5, 10 and 15 days treated groups when compared with the control. In *H. fossilis*, only 5 days treated group showed a significant difference ($p<0.01$) in comparison to its control group. The ERND activities in 10 days and 15 days treated groups showed a negligible response.

Table-1. Liver somatic index (LSI) of *C. punctatus* and *H. fossilis* exposed to dicofol (n= 6).

Liver somatic index [LSI %]

	C. punctatus	*H. fossilis*
Control	0.936±0.349	0.964±0.316
5 days	1.079±0.186*	1.110±0.194
10 days	1.161±0.126**	1.171±0.091*
15 days	1.177±0.140**	1.202±0.085**
F value	6.714	3.447

Values are the means ± SD. Means were analyzed using one-way ANOVA followed by Dunnett's test. Significantly different * ($p<0.05$), ** ($p<0.01$).

Table-2. CYP 450 content of *C. punctatus* and *H. fossilis* exposed to dicofol (n= 6).

CYP 450 content (nmole/mg protein)

	C. punctatus	*H. fossilis*
Control	0.288±0.091	0.338±0.157
5 days	0.318±0.085	0.433±0.072
10 days	0.401±0.107*	0.547±0.306
15 days	0.524±0.031**	0.713±0.397**
F value	12.992	4.551
P value	0.000	0.010

Values are the means ± SD. Means were analyzed using one-way ANOVA followed by Dunnett's test. Significantly different * ($p<0.05$), ** ($p<0.01$).

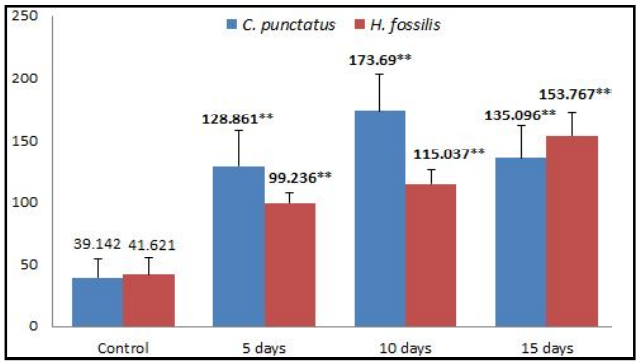

Figure 1. EROD activity (pmole resorufin formed/mg protein/min) of hepatic microsomes in *C. punctatus* and *H. fossilis* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Significantly different *($p<0.05$), ** ($p<0.01$)

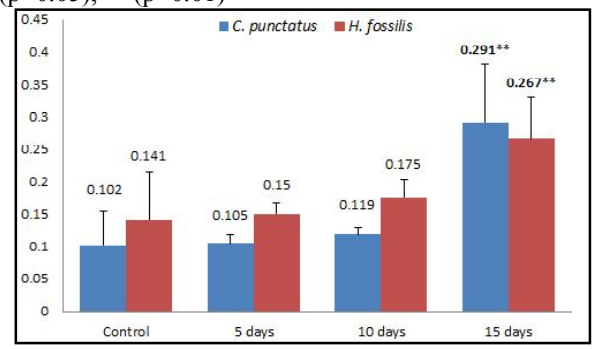

Figure 2. N,N-DMA activity (nmole formaldehyde formed/mg protein/min) of hepatic microsomes in *C. punctatus* and *H. fossilis* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Significantly different *($p<0.05$), ** ($p<0.01$).

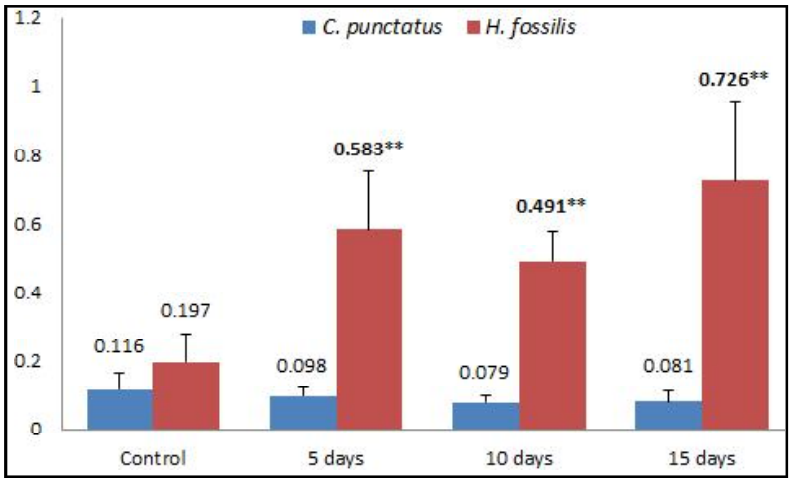

Figure 3. AH activity (nmole p-aminophenol formed/mg protein/min) of hepatic microsomes in *C. punctatus* and *H. fossilis* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Significantly different *($p<0.05$), ** ($p<0.01$).

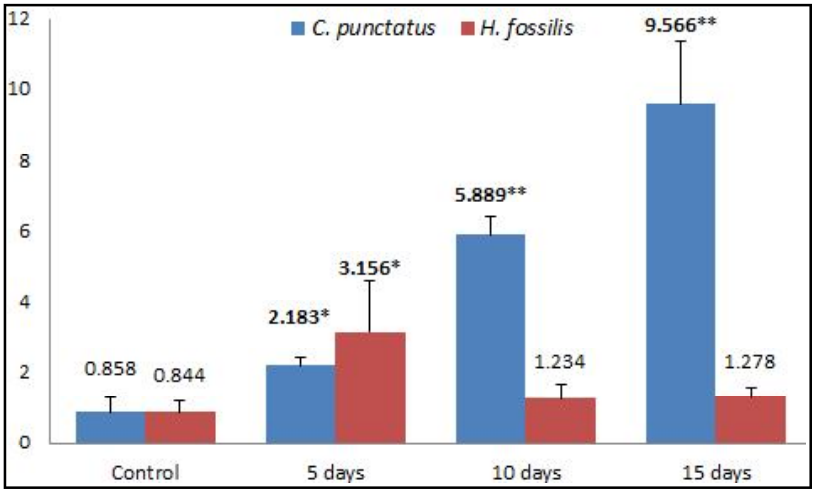

Figure 4. ERND activity (nmole formaldehyde formed/mg protein/min) of hepatic microsomes in *C. punctatus* and *H. fossilis* (n= 6). Means were analyzed using one-way ANOVA followed by Dunnett's test. Significantly different *($p<0.05$), ** ($p<0.01$).

The induction of CYP 450 is highly conserved and is present in numerous species besides mammals. The majority of CYP 450s in the liver are inducible CYP 450s (CYP1-CYP3). In most situations, ligand-induced activation of important receptor transcription factors, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and others, results in enhanced transcription and induction of CYP 450 [9].

Fish exposed to pollutants over longer periods of time, both in the lab and the field, frequently exhibit a rise in LSI [43]. The greater LSI value in experimental fish compared to control fish raises the possibility that pesticides may be to blame. LSI levels are often increased in vertebrates when hepatic microsomal CYP 450 is stimulated for the detoxification of organic substances, and the increase in LSI may be caused by the changing allotment of energy reserves for the process[21].

Due to their small molecular weight and lipophilic nature, organochlorine pesticides have the potential to activate either the aryl hydrocarbon receptor (AhR) or the pregnane X receptor (PXR), which would then promote the expression of genes encoding detoxification enzymes, particularly hepatic CYP 450 isoforms[1,6,8,36]. The CYP 450 family of catalytic enzymes are inducible when animals are exposed to toxins, which explains why experimental fish exposed to pesticides had higher concentrations of CYP 450 than the control group.

Only some of the CYP 450 subfamily enzymes, including CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1, and CYP3A, are involved in the metabolism of xenobiotics[11,24,25]. For assessing environmental risk assessment and environmental monitoring in response to organic pollutants and pesticides, CYP 450 is regarded as one of the most valuable and stable fish biomarkers[12,15]. According to studies by Lemaire *et al.*[127,26], exposure to DDT and dicofol increased the degree of EROD activity in fish liver microsomes. The best way to think of CYP1A-mediated EROD activity is as a measure of contaminant exposure rather than of effect. This biomarker may also be used as a forerunner in the assessment of contaminant risk[31]. The specific inducer of CYP2B in mammals has not been identified to induce CYP2B in fish, however exposure to the pesticide chlorpyrifos, dicofol, and cypermethrin has been associated with CYP2B-like activity in fish[2,3,26]. It is conceivable that certain CYP 450 has different roles in fish and mammals and use different induction methods[32]. It is also plausible that pesticides cause fish to exhibit CYP2B-like activities.

Despite the fact that CYP2E1 is involved in the metabolism of a number of low-molecular-weight xenobiotic substances, investigations have shown that exposure to the pesticides chlorpyrifos, dicofol, and cypermethrin stimulates CYP2E1 activity[2,26]. Nearly 50% of currently used pharmaceuticals, organic pollutants, and pesticides are metabolised by CYP3A, one of the most prevalent CYP 450 isoforms in fish liver. It has a wide range of substrate specificity[13,40]. Additionally, organic pollutants have been seen to stimulate the hepatic enzymes CYP2E1 and CYP3A4 in carp fish, which has been used as a sensitive biological indicator[15]. The CYP 450 enzyme may also be used by fish species as a very effective adaptive approach to boost their tolerance to pesticides and ensure their survival.

CYP 450 is an important xenobiotic metabolizing protein and is thought to serve a similar purpose in all vertebrate species[38]. After being exposed to methoxychlor, a structural equivalent of the DDT, channel catfish were reported to have their CYP1A, CYP2B, and CYP3A4 genes stimulated[40].

The induction of rat hepatic CYP1A, 2B, 2C, 2E, and 3A by dicofol and methoxychlor is also known[4,6,24].

The current work provides evidence of CYP 450 related xenobiotic metabolism in the liver of dicofol-exposed *C. punctatus* and *H. fossilis*. The involvement and presence of several isoforms for dicofol metabolism is also suggested by the stimulation of CYP 450 mediated catalytic activity through CYP1A, 2B, 2E1 and 3A, and these isoforms amply proved the use of CYP 450 as a crucial biomarker for monitoring the aquatic pollution.

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