**Original Article**

# Effects of Beta-Naphthoflavone and 3-Methylcholantheren on Biochemical Markers in Sturgeon Fish, *Huso huso*

*Katayoon Karimzadeh*[*]

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## ABTRACT

**Background:** Polycyclic aromatic hydrocarbons (PAHs) are a predominant group of contaminants that have been shown to accumulate and persist in marine organisms. We evaluated the dose-dependent induction of the cytochrome P4501A (CYP1A) system in the Sturgeon fish, *Huso huso* after after exposure to PAHs, beta-naphthoflavone (BNF) and 3-methylcholantheren.

**Methods:** The fish were treated by i.p. injections of beta–naphthoflavone and 3-methylcholantheren dissolved in corn oil at three doses 35, 70 and 105 mg/kg wet-body weight respectively for 72 h every day. Similarly, the control fish received injections of only corn oil. Microsomal fraction was prepared and ethoxy resorufin-O-deethylase activity (EROD) was measured in the fish liver. Cytochrome P4501A1 (CYP1A1) content was estimated by ELISA based on monoclonal anti-cod P4501A1.

**Results:** Beta-naphthoflovone and 3-methylcholantheren treated-fish developed 15-32 fold increase in Ethoxyresorufin-O-deethylase activity in liver microsomes. The results of ELISA showed higher levels of cytochrome P4501A content in treated fish as compared to controls.

**Conclusion:** A parallel increase of the CYP1A immunochemically assay and an increase in EROD activity could be recorded for beta –naphthoflavone and 3- methylcholantheren in injected fish. Dose-response relationships on the induction of EROD and CYP1A immunodetectable protein were recorded. Therefore, EROD activity and CYP1A content can be used as a biomarker of polycyclic aromatic hydrocarbons.


**Keywords:** 3-Methylcolantheren, Beta–Naphthoflavon, Biomarker, Ethoxyresorufin Deethylase, *Huso Huso*.

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## INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are represented as the most significant groups of environmental contaminants due to their carcinogenic and mutagenic potential [1, 2].

They are widely dispersed in aquatic environment through incomplete combustion of fuels such as gasoline, and release to the aquatic environment through atmospheric deposition and industrial effluent [1-4]. Since aquatic organisms are exposed to these pollutants, reduction in their reproduction and increased rate of mortality has been directly observed. Consequently, they can have adverse effects to human consumers [5, 6].

Chemical analyses of environmental samples are difficult, expensive and time-consuming method [6-8]. Analytical technique is unable to provide the risk assessment of contaminants and biological effects of xenobiotics after entering an ecosystem [5, 7]. Biomarkers have been explained as biological assay including as molecular, biochemical, cellular and physiological responses that can be determined in different parts of organisms such fluids, cells and tissues [7-9]. The use of biomarkers has more significant than chemical analysis. Therefore, biomarkers capable directly link exposure to contaminants with organism responses [6].

Increased levels of biomarkers can be measured after shortly exposed to or even absence of xenobiotics in aquatic environments [6]. The cytochrome P450 system is remarkable biomarker that responsible for removing of foreign compounds from organisms [6-10].

The cytochrome (CYP) heme containing protein are key detoxification enzymes in metabolic pathways of many xenobiotics

1. Department of Marine Biology, Islamic Azad University, Branch of Lahijan 1616, Lahijan, Iran.
*Corresponding Author: E-mail: karimzadehkatayoon@liau.ac.ir

[7].This group of enzymes could be involved in metabolic oxidation of wide range of endogenous substances including fatty acids, steroids and prostaglandins [11-13]. Among the cytochrome P450 monooxygenase system, cytochrome P4501A is used as biomarker of organic contaminants. CYP1A provided a key role in detoxification of environmental pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, (PCBs) and dioxins [3, 4]. Previous studies have revealed the fish CYP1A enzymes responds to xenobiotics in field and laboratory experiments, so inducibility of CYP1A is remarkable and sensitive biomarkers for exposing to organic pollutants such as PAHs and PCBs [6, 8, 9]. Thus, CYP1A induction has been used as biomarker for biomonitoring of those compounds in aquatic ecosystem. The induction of CYP1A enzyme can be measured by different methods. Enzyme activity assay using 7-ethoxyresorufin as substrate (EROD assay), CYP1A protein content using specific antibody (ELISA assay) and analysis the CYP1AmRNA using CDNA probe [14, 15]. Measurement of these activities has been suggested as possible means of monitoring environmental pollution [16, 17].

The aim of this study was to evaluate the effects of polycyclic aromatic hydrocarbon compounds, beta-naphthoflavone and 3-methylcholantheren on biochemical markers, 7-Ethoxyresorufin-0-deethylase (EROD) assay and cytochrome P4501A content in Sturgeon fish, *Huso huso*, and to determine the amount of induced cytochrome P450 1A protein content by an indirect ELISA method.

## MATERIALS AND METHODS

### *Chemicals*

Beta-naphthoflavone (BNF), 3-methylcholanterem (3-MC), Bovin serum albumin (BSA), 7-Ethoxyresorufin, Resorufin, Nicotinamide adenine dinucleotide (NADPH) was obtained from Sigma–Aldrich (Sigma Company, Germany). Horseradish peroxidase-conjugated anti-rabbit IgG, Tween 20, Sodium dodecyl sulfate (SDS) were purchased from Bio-Rad. Monoclonal cod P4501A was obtained from Bioscience Company. All the other chemicals were of analytical grade and were obtained from commercial sources at highest grade of purity available.

### *Fish*

Juvenile *H. huso* in the weight range of 252.40±3.82 g were obtained from the Shahid Beheshti Proliferation and Culture Center for Sturgeon fish, Rasht, Iran. The fish were transferred to the laboratory of Islamic Azad University, Lahijan Branch, Guilan Province, Iran. Six fish were selected for each concentration; in sum, 48 fishes in 4 groups for each polycyclic aromatic compound. All fishes were acclimated for two weeks before the assays.

The fish received i.p. injections of BNF and 3-MC in corn oil with three different doses, 35, 70 and 105 mg/ml kg fish body weight for 72 h every day. The control fish only received the corn oil. After exposure, all the fish were anesthetized with ice at the same day and decapitated. The livers were removed and were placed in liquid nitrogen and transported until assessment.

### *Microsomal Fraction Preparation*

Microsomes were prepared from fish liver by differential centrifugation. The microsomes were washed by suspending them in 1.15% KCl containing 1mM EDTA and collected by centrifugation, and the pellets were resuspended in 10% glycerol containing 1Mm EDTA and were stored in liquid nitrogen until use [15,18 ].

### *Protein Quantification*

Total protein concentrations of each microsomal fraction were quantified by the method proposed by Lowry et al. [19] using bovine serum albumin as standard solution.

### *Enzyme Assay*

Seven-Ethoxyresorufin-O-deethylase (EROD) activity was evaluated at 25 °C by measuring the fluorescence of the deethylated product, resorufin, following the Burke and Mayer's procedures [20, 21]. Reaction mixtures were prepared in 96-well plates by adding 190 µL phosphate buffer solution (pH=7.8), 2µL 7-ethoxyresorufin working solution (5 µM), and 5 µL of microsomes to each well. Samples were thoroughly mixed for 10 s using a microplate shaker to allow binding of substrate with the enzymes. Then, 5 µL NADPH (30 mM) was added to each well to initiate the reactions. Following another 10 s of medium shaking,

resorufin production was quantified fluorometrically (excitation at 530 nm and emission at 590 nm), at 1 min intervals for a total of 16 min.

### *CYP1A Protein Assay*

Microsomes were diluted to a concentration of about 10 µg /ml in 50 mM bicarbonate buffer with pH 9.5, and adsorbed to microliter wells for two hours at 37 ºC at 100 µl per well. After washing the wells, the wells were blocked for unspecific binding with 200µl 5% Tween 20 in Tris -buffer (TBS) for 20 min at room temperature. Primary antibody (monoclonal anti-cod P4501A1) was diluted to 1:10000 in TBS and was added to the wells (100 µl), and was incubated for two hours at 37 ºC. After washing four times with Tris–buffer with tween (TBST), 100 µl of secondary antibody (GAR-HRP) at a 1:6000 dilution in TBS was added to each well, and incubated for one hour at 37 ºC. The last washing was repeated six times, and then 100 µl of TMB substrate was added to each well. The reaction was developed for 10-30 min and was stopped by addition of $H_2$ $SO_4$ to each well [15, 22].

### *Statistical Analysis*

Statistical analyses of data were carried out with SPSS 17.0 software package (Chicago, IL, USA). Significance of results was tested by an analysis of variance (ANOVA) and Duncan's Multiple-Range Test. Significance of differences was defined at $P< 0.05$.

## RESULTS

EROD activity trends, following injection of 3-methylcholanthern and beta naphtoflavone are shown in Figure 1.There was significant differences between controls and the treated fish (32 fold increases in EROD activity) ($P<0.05$). The highest increase in EROD activity occurred with the maximum injection dose (105 mg/kg wet-body weight) of 3-methylcholanthern. Similar results were obtained with beta - naphtoflavone. *Huso huso* CYP1A protein content following i.p. injection of 3-methylcholantheren and beta naphtoflavone are illustrated in Figure 2 and 3, respectively. The highest cytochrome p4501A protein content was observed in fish treated with 3-methylcholantheren. Similarly, CYP1A protein content was increased significantly after the second dose of beta-naphtoflavone (75 mg/kg wet-body weight) was applied. Additionally, there was no significant difference between the medium and high doses of beta – naphtoflavone.

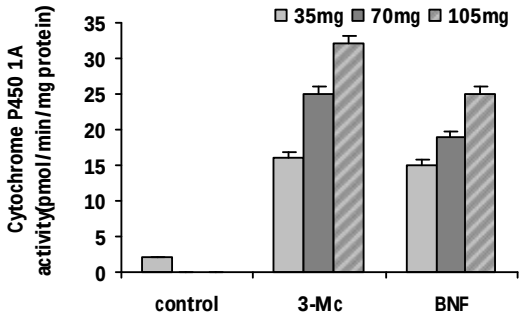


**Figure.1.** Effect of different concentration of 3-Methylcholantheren and Beta-naphtoflavone on cytochrome P4501A activity (EROD activity) in Sturgeon fish (*Huso huso)*. The results are mean ±SDE; n=6.Significantly different from controls.

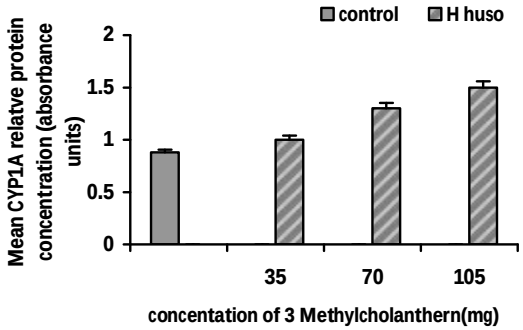


**Figure. 2.** Effect of different concentration of 3-Methylcholantheren on cytochrome P4501A content in Sturgeon fish (*Huso huso*) using monoclonal Cod P4501A. The results are mean ±SDE; n=6.Significantly different from controls.

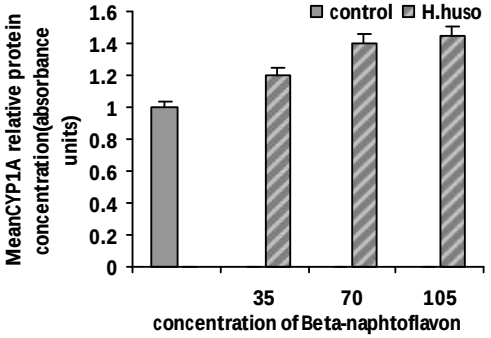


**Figure. 3.** Effect of different concentration of Beta-naphtoflavone on cytochrome P4501A content in Sturgeon fish (*Huso huso*) using monoclonal Cod P4501A. The results are mean ±SDE; n=6.Significantly different from controls.

## DISCUSSION

Induction of cytochrome P4501A as protein expression synthesis in respond to polycyclic aromatic hydrocarbons such as 3-methylcholanthern and beta-naphthoflavone compounds in different fish species have been previously observed [6, 14, 15, 21, 23, 24]. In this study, treatment of *Huso huso* with these compounds has resulted in induction of liver EROD activity, associated with cytochrome P450 1A protein content. Dose-related activity and levels of cytochrome P4501A in Sturgeon exposed to BNF and 3-methylcholantheren were indicated, and both EROD and ELISA values increased with increasing dose of exposures. Cytochrome P4501A1 levels showed lower responsiveness when compared to EROD activity measurements after 3-methylcholantheren treatment in *Huso huso*. Higher level of CYP1A protein content using monoclonal cod P4501A (ELISA technique) was recorded in beta-naphthoflavone treated Sturgeon fish, *Acipenser persicus* [15, 21]. Since BNF has been reported as a specific inducer of CYP1A and the monoclonal antibody reacted only with a single protein band (54-59 kDa ) in Western blots prepared from microsomal preparations of livers of BNF-induced fish [15, 21, 22, 24]. Formation of new cytochrome P450 1A with an apparent molecular weight of about 58kDa on electrophoresis pattern and significant induction of EROD suggested that PAHs induce cytochrome P450 1A in Sturgeon liver microsomes (data are not shown) [21].

Moreover, we found a lower induction factor in CYP1A content (1.6 fold), while EROD activity of liver microsomes increased by factor 25-32 for highest doses of BNF and 3-MC respectively. Despite this lower sensitivity of ELISA technique, when defined as an increase of response per increase of dose, the ELISA offers the advantages. ELISA technique is simple and rapid test for simultaneous screening of a large number of samples in comparison with enzyme activity, and it (immunochemical detection) is less sensitive to storage conditions of CYP1A protein [14, 22]. Generally, EROD activity and CYP1A content can be used as biochemical markers to monitor aquatic contaminants.

## CONCLUSION

The results of this study demonstrate that polycyclic aromatic hydrocarbons such as beta-naphthoflovone and 3-methylcholanthern have the ability to induce P4501A activity. Induction mechanism involves elevation of microsomal protein expression from *Huso huso* cytochrome P450 1A in which was established in the cytochrome P4501A content using ELISA assay.

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