# Application of Cytochrome P 450 (CYP1A) as Biomarker in Fish to Evaluate Aquatic Contamination and its Current Status in Thailand

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## Abstract

Cytochrome P450 (CYP1A) is monooxygenases enzyme playing important roles in metabolizing (activation and/or inactivation) xenobiotics in the organism which is obtained by various routes such as ingestion or skin absorption, lung and other epithelial layers contacting with the surrounding environment. In the aquatic environment contamination with xenobiotics such as polynuclear aromatic hydrocarbons (PAH), planar polychlorinated biphenyls (PCB) and dioxins, CYP1A expression in fish can be evaluated by measuring 7-Ethoxyresorufin O-Deethylase (EROD) activity or antibody techniques such as Western blotting, Enzyme Linked Immunosorbent Assay (ELISA) or other immunohistochemistry. Although P450 enzymes mostly found in the liver, they also play a role in other extra hepatic organs of vertebrates ranging from fish to mammals. Different expression of P450 enzyme among various organs and cell types should be concerned in studying the responses of those cells and organs to toxicants. As the importance described above, CYP1A can be used as the useful alternative bio-indicator to assess water pollution. In Thailand, there have been few studies on CYP1A application as a biomarker. Thus, its efficiency in water monitoring should be further studied.

**Keywords:** Bioindicator; Benzo[a]pyrene; Monooxygenase; Xenobiotic

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## Introduction

A biomarker is easily described as an alteration in biological, physical or physiological; ranging from molecular to cellular, responding to chemicals or toxicants exposure [1]. The alteration occurred in fish which is caused by toxicants exposure may be applied to identify the contamination status in the environment. Because the fate of xenobiotic substances is quite complex and cannot be studied by using simple partitioning models, it should concern toxicokinetics, metabolism, biota-sediment accumulation factors, organ-specific bioaccumulation and bound residues. For fulfilling this gap of knowledge, it may require sophisticated models to analyses toxicant fate in the organism [2].

The fate of toxicant in organism can be divided into two dominant pathways; detoxication and harmful or bioactivation. The toxicant may be biotransformed in the liver. The transformation process can be classified into three phases. Phase I consists of oxidation, reduction or hydrolysis of the original molecule. Then, the transformed molecule can be conjugated in phase II and catabolized in phase III [3]. Phase I of biotransformation metabolism is classified as the very important step because it determines the acceptance level of toxicant exposed. This step consists of oxidation, reduction or hydrolysis which unmasks or adds active functional groups [4]. Many xenobiotic compounds are transformed by microsomal monooxygenase (MO) enzymes or known as the mixed-function oxidase (MFO) system such as cytochrome P450 (CYP1A), cytochrome b5 (CYPB5), and NADPH cytochrome P450 reductase (P450 RED) in this step. The dominant catalyst playing in phase I in fish are cytochrome P450-dependent MOs. They belong to the family of heam proteins which are membrane-bound proteins mostly found in the endoplasmic reticulum of the liver [2], [5] - [6]. From the importance described above, monooxygenase enzyme especially CYP1A has been applied as bio-indicator to monitor xenobiotic contamination in aquatic environment because CYP1A is induced in the liver for transforming toxicant structure and excreting [7] - [8].

## Induction of CYP1A

Cytochrome P450 (CYP1A) is a heme-thiolate enzymes playing an important role in the biotransformation of endogenous and exogenous compounds. Thus, it can be used as a biomarker for monitoring pollution status in the environment especially in the toxicant exposure issue. CYP1A production is activated after its specific receptor induced by xenobiotic; aryl hydrocarbon. In the cell, xenobiotic which acts as ligand attaches to aryl

receptor (Ah-receptor, AHR) resulting in CYP1A production. The samples of inducing agent for CYP1A are PAHs, PCBs, TCB and dioxin [9] - [10]. After ligand attaches to Ah-receptor to become ligand-Ah receptor complex, heat shock protein (Hsp 90) is released and then the ligand-Ah receptor complex being bound to translocating protein in cytoplasm making the complex enter cell nucleus. After that, it interacts with xenobiotic response elements (XREs) inducing CYP1A gene to produce CYP1A mRNA and then protein synthesis. Next, apoprotein binds with heam and being transported to endoplasmic reticulum membrane for metabolizing toxicants (Figure 1) [8], [11]. Hahn, M.E. et al. [12] reported that CY1A enzymes can be induced by xenobiotics which is specific to receptor. This phenomenon can be found in all vertebrate.

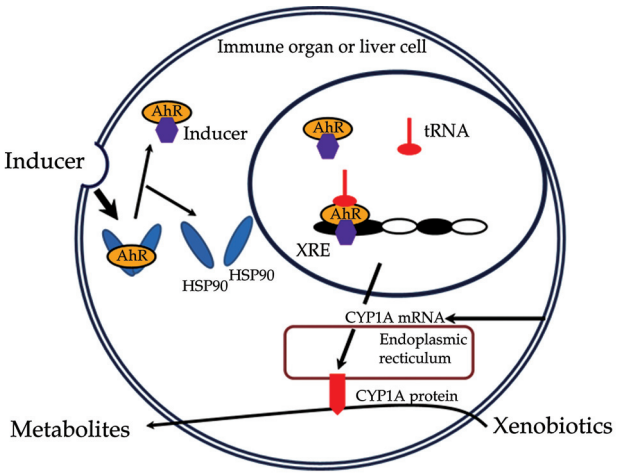


**Figure 1** Synthesis of CYP1A in liver cells [13]

In the fish, CYP1A synthesis is induced by many xenobiotics and occurs in many organs such as liver, gill, kidney, heart and gastric mucosa [14]. The induction is influenced by many factors such as time, dose, route and other surrounding factors [8]. For examples, Carlson, E.A. et al. [15] reported that BaP had no acute effect. Moreover, in 2005, revealed that *Sebastiscus marmoratus* fish which exposed to 200 mg/kg BW of BaP had no effect or noticeable effects in their behavior in 7 days after exposure. Rees, C.B. et al. [10] found that Atlantic salmon in destructive sampling site was induced to produce CYP1A mRNA. The amount of mRNA in exposed fish was 12-fold higher than that in the controlled group after one day of exposure. After two days, BNF-induced kept rising until 85-fold compared to the control group. The intra-peritoneal injection of 50 ug $g^{-1}$ h-naphthoflavone (BNF) could cause acute effect because of its lipophilic properties [16].

## Functions and metabolite of CYP1A

Metabolic processes are the important responding mechanism of the organism consisting of bioaccumulation, biomagnification, and toxicokinetics. The cytochrome P450 monooxygenases (CYPs) is the enzyme involved in the oxidative biotransformation (phase I) of a wide range of xenobiotic and endogenous compounds [5], [17]. The intermediated products of phase I are then conjugated to larger endogenous molecules in phase II controled by enzymes such as glutathione-S-tranferase (GST) and uridine diphosphoglucuronosyl transferase (UDPGT) [18] - [19]. The enzymes involved in phase I and II are mostly found in hepatic tissues. They transform lipophilic compounds into detoxified or bioactivated forms depending on each specific mechanism and xenobiotic substance. CYP isozymes are qualitative and quantitative differences among species and populations in each species [20] - [21]. Their inductions can be used to determine the ability of exposed animals to metabolize toxicant they are exposed to [22].

## Detection of CYP1A

CYP1A induction was firstly applied to monitor toxicant exposure in fish in the mid1970s, and then this biomarker was characterized by many researchers such as Stegeman, J.J. and Hahn, M.E. [5] and Bucheli, T.D. and Fent, K. [6]. In addition, CYP1A induction is wildly used to determine the distribution and levels of CYP1A inducers in the environment.

Nowadays, CYP1A enzyme can be measured in both direct and indirect methods. For the direct, it is performed based on immune detection which apply to the specification of antibody and CYP1A. The advantages of this method are high sensitivity, specificity and applying with many samples. With label tracking tag, it can be used to measure interaction in both qualitative and quantitative. In addition, it can also be applied in Western blotting on nitrocellulose and PVDF or in microtiter plate (ELISA) [23]. However, the interaction of antibody and CYP1A may cross react to inter and intra species which is the limitation of this method. Thus, it must be improved to monitoring vitellogenin in various fish species by using polyclonal antibody (PAb) or monoclonal antibody (MAb) which specify to its CYP1A. Moreover, the appropriate condition of antibody should be identified.

For the examples, CYP1A can be measured by using ELISA technique which Sturve, J. et al. [24] suggested that hepatic CYP1A levels in eelpout after exposed to crude oil was significantly increased comparing to the control group. For Western blot

technique applied in rainbow trout after exposed to benzo-a-pyrene in the study of Ortiz-Delgado, J.B. et al. [25] found the expression of CYP1A as dark brown band in the Figure 2.

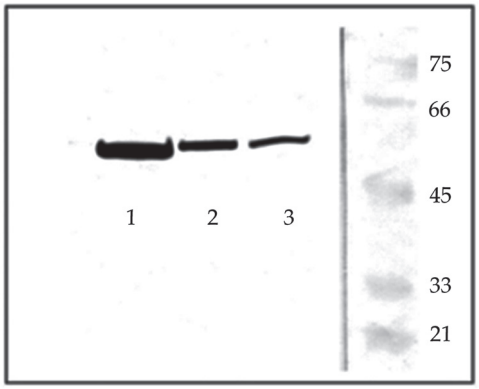


**Figure 2** CYP1A protein in brain and liver from control and exposed specimens (20 days of treatment) measured by Western blot technique. The amount of protein applied to each lane was 20 μg. Liver of rainbow trout (*O. mykiss*) was used as a positive control. (Lane 1: hepatic microsomes from 100 μg B[a]P/L *S. aurata* exposed organisms; Lane 2: liver from rainbow trout; Lane 3: microsomes from whole brain homogenates of B[a]P exposed fish; Right lane: molecular weight marker) [25].

Immunohistochemical analysis is another technique applying to test CYP1A expression based on the function of antibody as same as in ELISA and Western blot technique. Zodrow, J.M. et al. [14] suggested that immunohistochemical analysis is a useful technique to localize CYP1A expression in multiple organs. The result showed positively CYP1A expression in liver, gill, kidney, heart and gastric mucosa of zebrafish as shown in Figure 3.

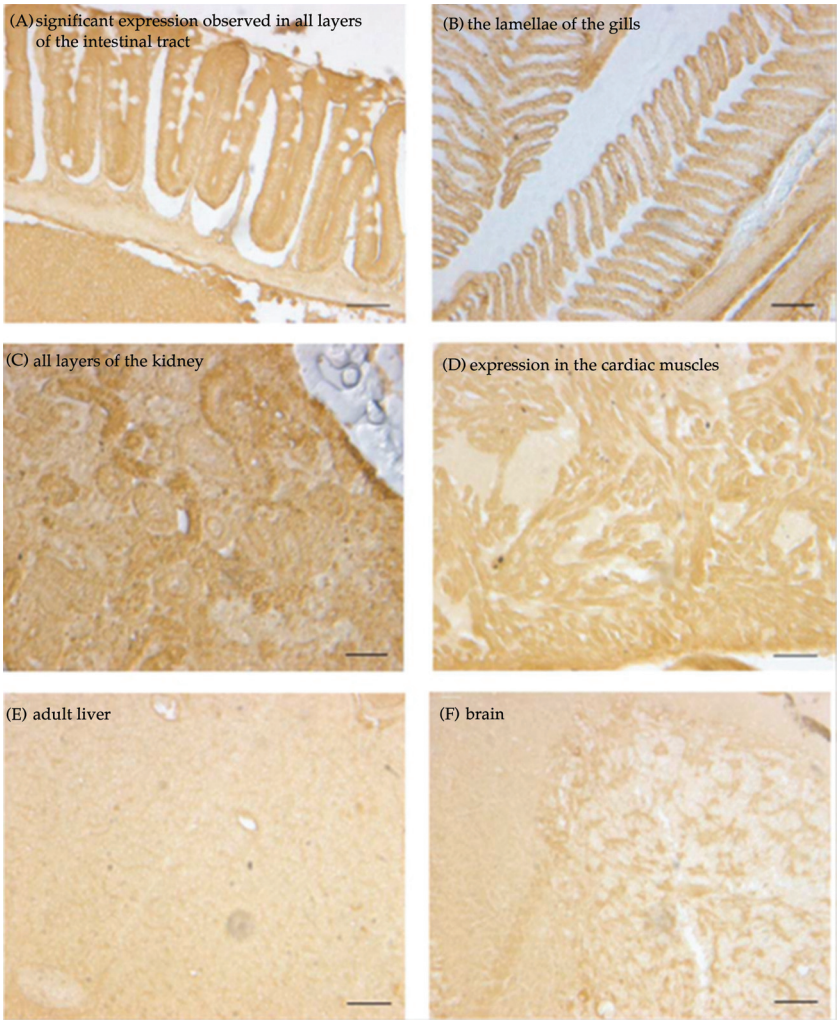


**Figure 3** In situ immunohistochemical alteration in adult zebrafish after exposed to 70 ng/g fish of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). The bright field images were digitally acquired using a 20× objective and the scale bar is 50 $\mu$m [14]

The amount of CYP1A enzyme final product can be directly measured and CYP1A interaction by using fluorescence spectrophotometer. The substrates are ethoxyresorufin and benzo[a]pyrene, however, ethoxyresorufin is mostly used and thus called that EROD assay (Ethoxyresorufin O-de-ethylase assay). In addition, EROD activity is induced by direct contact of organism tissue and toxicant in the water. The method is quite easily performed, the process for preparing microsomes which normally time-consuming in EROD assays is avoided [26]. For the example, a method measuring EROD activity

in intact gill filament tips of rainbow trout was developed by J$\ddot{o}$nsson et al. [27]. Moreover, EROD assay can be applied to measure CYP1A enzyme in many organs such as Sturve, J. et al. [24] applied it to study the effects of an oil spill in liver of eelpout at the concentrations of 10, 100 and 1,000 $\mu$g/L. They found a dose-dependent elevation after exposure to the crude oil in the laboratory. The higher doses resulted in significant increasing in EROD activities compared to the control. The levels of EROD activity increasing in low, medium, and highest dose were 3, 18, and 72 times, respectively. In the study of McNeill, S.A. et al. [22] on the impacts of oil contaminated sand on rainbow trout collected from Mildred lake, South Bison Pond and Demonstration Pond, the results showed that liver hepatic EROD activity in the fish from contaminated site was two-fold and three-fold higher than that in non-contaminated site.

For applying in the field study, EROD activity measurement can be effectively used as the study of Parente, T.E.M. et al. [28] which assessed CYP1A expression associated with EROD activity in liver of the of the loricariid catfish. And, EROD activity was not detectable in control fish. However, the activity was detected in microsomal liver of *Ancistrus* sp. and *Corydoras* sp. after exposed to the PCB126 by both intra peritoneally or skin absorption which applied in the controlled fish as same as in the exposed fish.

Moreover, CYP1A mRNA and CYP1A gene expression in xenobiotic exposed fish can also be used. In 2005, Rees, C.B. et al. [10] revealed that Atlantic salmon collected from destructive site showed CYP1A mRNA induction. The inductive expression was 12-fold higher than that of the controlled fish. The trend of increasing kept continuously until 2 days after exposure where BNF-induced was 85-fold higher than the control.

The measurement of CYP1A expression in fish can be deployed by many methods as mentioned above depending on research objective, instrument, sample character, time requirement and expertise. Specifically, Stegeman, J.J. [29] and Rees, C.B. et al. [10] identified that CYP1A is a useful biomarker for applying in both laboratory and field conditions. Table 1 presents the examples of CYP1A measuring method which applied to various fish species.

Table 1 The examples of measuring method for CYP1A in fish

| References | Xenobiotics | Species | Methods | Organs |
|---|---|---|---|---|
| Zodrow, J.M. et al. [14] | TCDD | zebrafish (*Danio rerio*) | Immunohistochemistry | liver, gill, kidney, heart, gastric mucosa |
| Rees, C.B. et al. [10] | B-napthoflavone (BNF) | atlantic salmon (*Salmo salar*) | Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) | liver |
| Hassanin, A.A.I. et al. [30] | Benzo[a]pyrene | tilapia (*Oreochromis niloticus*) | Reverse transcriptase-assisted polymerase chain reaction (RT-PCR) | liver |
| Klemz, C. et al. [31] | Field study (Marumbi River in the city of Morretes Paranǎ, Brazil) | catfish (*Ancistrus multispinis*) | Differenntial visible spectroscopy and Western blotting | liver |
| Parente, T.E.M. et al. [28] | PCB, BNF | catfish (*Pterygoplichthys* sp.) | EROD activity, PCR | liver |
| McNeill, S.A. et al. [22] | Oil-sands-affected water | rainbow trout | Ethoxyresorufin-O-dethylase (EROD) activity | liver |
| Sturve, J. et al. [24] | Oil spill | eelpout (*Zoarces viviparus*) | Ethoxyresorufin-O-dethylase (EROD) activity and Enzyme-linked immunosorbent assay (ELISA) | liver |

## Current status in Thailand

As generally known, many xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), dioxins, dibenzofurans and polychlorinated biphenyls (PCBs) wildly contaminate in the environment. After entering the organism, they can induce cytochrome P4501A (CYP1A) production which can be used as warning signal for toxicant especially carcinogen exposure. This application can also be performed to monitor health status of aquatic organisms [30]. Many areas in Thailand are having the problem of water contamination. Thus, many researchers have applied CYP1A assessment as a key bio-indicator for environmental monitoring. In 2007, Cheevaporn, V.F. and Beamish, F.B.H. [32] found the higher cytochrome P450 1A activity in liver and bile of the tonguefish collected from petroleum hydrocarbons contaminated area compared to the pristine area. PAH concentration in sediment collected from oil spilled and referenc areas were 5.03 ± 0.42 and 0.21 ± 0.043 $\mu g^{-1}$ dry weight, respectively.

In the study of Kachanopas-Barnette, P. et al. [33] which characterized the molecule of cytochrome P450 1A (CYP1A) in Asian sea bass (*Lates calcalifer* Bloch) revealed that polyclonal antibodies (PAb) was induced by Asian sea bass CYP1A in mice. After western blot analysis was performed the results showed that PAb recognized CYP1A protein produced in Asian sea bass after exposure to benzo[a]pyrene (BaP). CYP1A orthologue induction was isolated and confirmed by partial cDNA which used a reverse transcriptase polymerase chain reaction (RT-PCR) approach. The results of quantitative RT-PCR analysis showed that CYP1A mRNA in fish injected with BaP was 2.5-fold higher than that in non-injected. This results indicated that the coastal area in Si Racha and Ao Udom is highly contaminated with PAH compared to Laem chabang which confirms the studies performed in other temperate waters area in Thailand.

Polyclonal antibody against Asian sea bass is suitable in measuring CYP1A in different tropical fish species. Because of this advantage, in 2014, Nanthanawat, P. et al. [34] performed comprehensive study to produce monoclonal antibody against P450 (CYP1A) in Asian sea bass (*Lates calcarifer* Bloch) after exposure to B[a]P. The selected clone against CYP1A was extracted from Asian sea bass. Their molecular weight was 56 and 74 kDa. The results of dot blot and Western blot identified that there are 3 monoclonal antibody against CYP1A. Moreover, obtained monoclonal antibody can cross react with some marine, estuarine, and fresh water fish.

CYP1A has been studied not only in protein form but also in gene level. In Thailand, it has been performed in Asian sea bass because of its high economic importance. The study of Ngamdee, V. and Boonpakdee, C. [35] cloned and sequenced the DNA fragment of CYP1A gene isolated from Sea bass (*Lates calcarifer*). This DNA fragment was isolated from liver and then amplified by PCR (Polymerase Chain Reaction) technique. The sequence of partial CYP1A gene was highly similar to Sea perch (*Lateolabrax japonicus*). The structure of CYP1A gene consists of 3 exons (134, 88 and 126 bp) and 2 introns (91 and 210 bp). Its partial structure and sequences can be applied as bio-indicator to assess gene expression levels responding to PAHs exposure. In addition, EROD activity assessment is a technique monitoring xenobiotic exposure which induce CYP1A expression. Found high level of CYP1A expression in fish exposed to chlorpyrifos or carbaryl.

From the information mentioned above, the efficiency in CYP1A application as bio-indicator was achieved in lab condition. Its gene sequence identification and antibody production can also be performed. In the future, the efficiency of CYP1A indicator should be studied in field condition for applying in water resource and biodiversity management.

## Conclusion

The impact of toxicant on the health status of aquatic organisms can be assessed by using biomarkers. The induced CYP1A expression may be used as useful alternative bio-indicator for PAHs, planar halogenated aromatic hydrocarbons (PHAHs), planar polychlorinated biphenyls (PCBs) and dioxins exposure. For aquatic environment, the appropriate fish such as native species can be applied as bio-indicator monitoring CYP1A inducer. The selection of method for measuring CYP1A expression based on the objective, instrument, type and amount of sample, and expertise. In Thailand, the application of CYP1A measurement has not been sufficient to assess the contamination status because the studies we found have been conducted in only eastern part of Thailand. Moreover, some of them have been performed in lab condition. Thus, it should be intensely studied for improving contamination assessment procedure and management.

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