EnvironmentAsia
The international journal published by the Thai Society of Higher Education Institutes on Environment

Available online at www.tshe.org/EA
EnvironmentAsia 3(2) (2010) 8-14

Polycyclic Aromatic Hydrocarbon Contamination in Nile Tilapia (*Oreochromis Niloticus*): Analysis in Liver and Bile

Voravit Cheevaporn [a], Montira Pindang [a] and Herbert F. Helander [b]

[a] *Department of Environmental Science, Faculty of Science, Rambhai Barni Rajabhat University, Chantaburi 22000, Thailand*
[b] *Department of Gastroresearch, Sahlgren Academey, Göteborg University, Sweden*

Abstract

Polycyclic aromatic hydrocarbons (PAHs), mainly from petroleum products, are a source of worldwide contamination, and it is in the present study, we exposed Nile Tilapia in aquaria to No-Observed-Effect-Levels (NOELs) of naphthalene, phenanthrene and pyrene for periods up to 9 days in a continuous flow system. Additional studies were carried out on fish exposed to lubricating oil, gasoline and diesel oil. Two methods were used to measure the levels of these PAHs: determination of ethoxyresorufin-O-deethylase (EROD) activity in liver extracts, and fixed wavelength fluorescence (FF) of PAH in bile. Optimal excitation wavelengths for FF analyses were determined to 290, 260 and 341 nm for naphthalene, phenanthrene and pyrene, respectively. The optimal emission wavelengths were 335, 380 and 383 nm, respectively. EROD activity and fluorescence intensity increased with increasing PAH concentrations and increasing exposure times. Similar results were obtained after exposure to lubricating oil, gasoline, or diesel oil. There was a high and significant correlation between the two methods. In view of its higher accuracy, lower cost, and convenience FF offered better possibilities than EROD determination to monitor PAH contamination in fish.

Keywords: polycyclic aromatic hydrocarbons; fixed wavelength fluorescence; nile tilapia; naphthalene; phenanthrene, pyrene

1. Introduction

Contamination of the aquatic system by polycyclic aromatic hydrocarbons (PAH) is a major ecological problem. Whereas our use of these hydrocarbons mainly takes place on earth, it is the rivers, lakes, and ultimately the oceans which serve as the final reservoirs for these pollutants after we have used them. It is obvious, that all plants and organisms living in water will be affected.

Against this background, it becomes important to choose a rapid, inexpensive, technically simple, yet sufficiently accurate, method to monitor the degree of PAH pollution, a method which can easily be utilized also outside of a research laboratory. In the present study, we compared two previously described methods for monitoring such contamination in fish, *viz*., fixed wavelength fluorescence (FF) of bile metabolites of PAH, and determination of Ethoxyresorufin-O-Deethylase (EROD) activity in liver extracts from Nile Tilapia (*Oreochromis niloticus*). This enzyme plays in important role in the CYP450 1A pathway for detoxification in the liver.

2. Materials and Methods

The fishes were collected from a pond at Chonburi Inland Fishery Research and Development Center, Chonburi, Thailand. They were about 3-4 months old, weighed about 50 grams and were about 7.5–10 cm long. They were acclimatized in an aerated plastic tank for 14 days, the tank contained about 6 m^3 of fresh water. They were fed with pellet food and were fasted for 1 day before experiment.

The fishes–usually 30 at a time-were then transferred to a 50-L glass tank with continuous water flow system; 4% of the water was changed every day. Dissolved oxygen ranged 4.3–5.3 mg/L, pH was 7.5–8.0, and the temperature was 27.8–29.9°C; data were recorded daily. Six fishes were selected for each of the treatments. Before the main studies, the LC_{50} -96 hr and No Observed Effect Concentration (NOEC) were determined for naphthalene, phenanthrene and pyrene, and based on the results (Table 1), the subletal concentrations to be used in the main studies were estimated by probit regression and were selected as the sublethal concentrations for the study (Finney, 1971).

During the main studies, the fishes were kept in the glass tank for 6 hr, 1 d, 5 d, or 9 d, while they were exposed to different concentrations of the PAHs, or commercial oils (Table 2).

2.1. Determination of Ethoxyresorufin-O-Deethylase (EROD) activity and protein concentration in liver

Table 1. Results from toxocity tests (μM).

	LC_{50} (96 hr)	NOEC	Sublethal conc.
Naphthalene	71.3	55.1	0–51.9
Phenanthrene	28.0	19.6	0–16.8
Pyrene	3.96	1.48	0–1.24

Table 2. PAH concentrations during exposure.

	units	Concentrations	No. of testing fishes
Naphthalene	μM	0, 0.81, 3.42, 13.0, 51.9	150
Phenanthrene	μM	0, 0.24, 1.05, 4.21, 16.8	150
Pyrene	nM	0. 19.8, 79.1, 312, 1240	150
Lubricating oil	ml/L	0 and 1	120
Gasoline	ml/L	0 and 1	120
Diesel oil	ml/L	0 and 1	120

At the end of the exposure period the fish were killed and weighed. The liver was then removed, and using a syringe with a needle some of the bile was collected from the gall bladder. Pieces of the liver were put into plastic vials, homogenized for one minute in a homogenizing buffer pH 7.5, 10 times the weight of the liver samples, centrifuged at 10,000 rpm for 20 min at 4°C.

The S9 supernatant was collected for determination of ethoxyresorufin-O-deethylase (EROD) activity according to Kennedy and Jones (1994). Briefly, fluorescence was recorded every minute between 2 and 10 min in a fluorescense microplate reader (Wallac Victor V 1420) using 530 nm excitation wavelength and 590 nm emission wavelength; Total protein analysis was conducted with 100 µl of tissue homogenate from each fish using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Life Sciences Group, Hercules, CA, USA). Colorimetric analysis was performed following the standard procedure outlined in the protein assay instructions. Bovine serum albumin (BSA) was used to obtain a standard curve from which relative measurements of protein concentration in the samples were made (Bradford, 1976).

2.2. Fixed wavelength fluorescence (FF) of bile metabolites of PAHs

For the analysis of naphtalene, phenanthrene and pyrene metabolites in bile sample by fluorescence screening, we determined the optimal excitation and emission wavelengths in a JASCO FP-6200 spectrofluorometer. The publications by Lin *et al*. (1996) and by Shailaja *et al*. (2006) served as a basis for this part of the study. We then determined PAH metabolites in the bile from the fish which had been exposed to either of these PAHs or commercial oils. The bile was diluted 1:2,500 in 48% ethanol.

We confirmed the optimal wavelength pairs for FF detection of naphthalene, phenanthrene and pyrene with peaks at 290/335, 260/380, and 341/383 nm (Fig. 1).

3. Results

Our study (Figs. 2-4) showed that the fluorescence levels of PAHs metabolites and EROD activity in fish exposed to naphthalene, phenanthrene or pyrene increased with the increasing of PAH concentrations and exposure time (up to 9 days). Exposure to commercial oils, including lubricating oil, gasoline, and diesel oil, which contain an abundance of PAHs produced similar increases in the fluorescence levels of PAHs metabolites and in EROD activities.

A high correlation ($p=0.01$) was found between fluorescence levels in bile and EROD activities in livers from the same fishes which had been treated with PAHs (Fig. 3). The correlation coefficients (R values) were 0.724, 0.655, and 0.731 for naphtalene, phenathrene, and pyrene, respectively.

When correlating fluorescence levels in bile (excitation wavelength 260 nm, emission wavelength 380 nm, corresponding to phenanthrene) with EROD activities in livers from fish which had been treated with lubricating oil, gasoline, or diesel oil, the R values were 0.547, 0.762, and 0.580 ($p=0.01$), respectively.

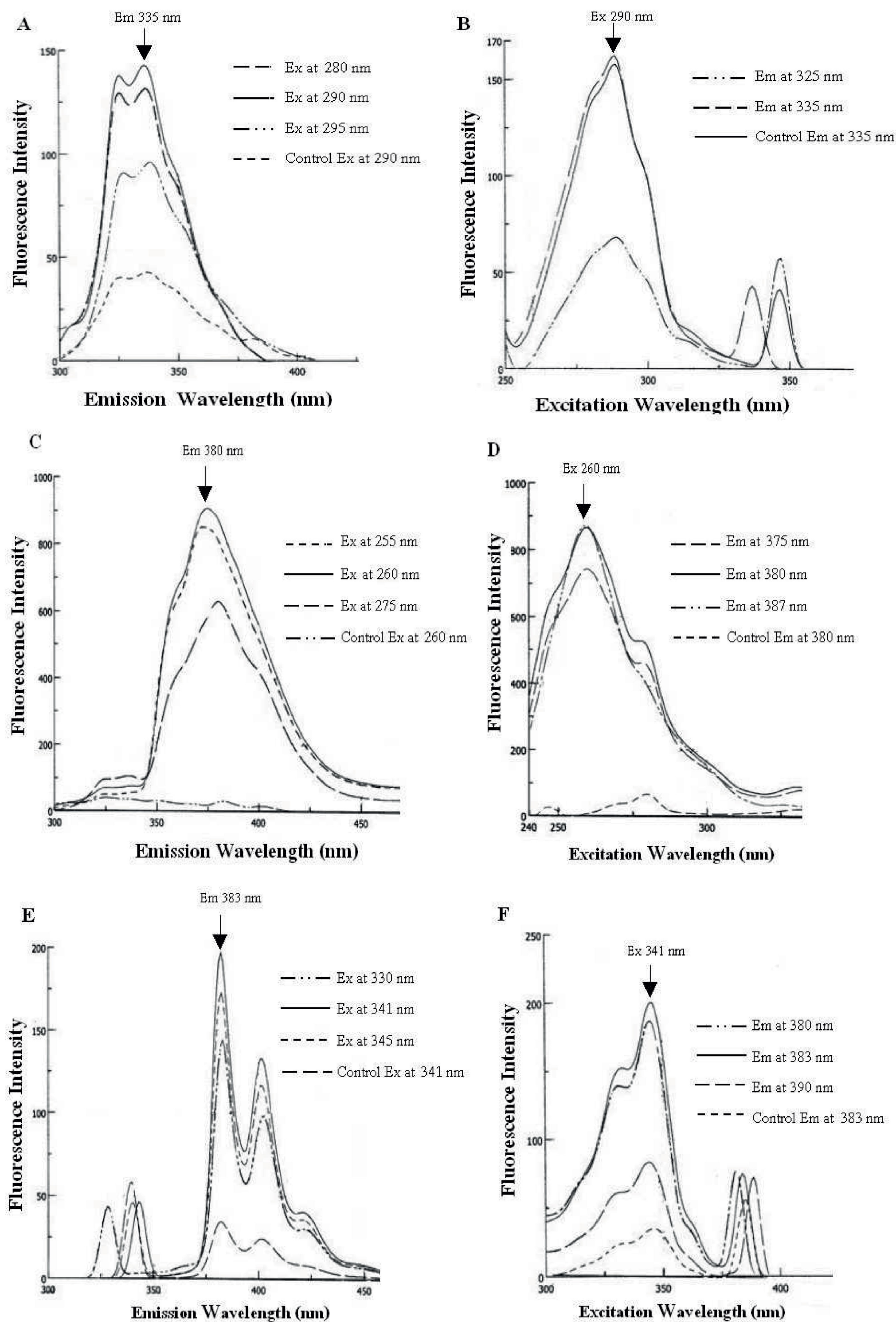

Figure 1. Fluorescence screening measurements (means; $n=5$) for determination of optimal excitation/emission wavelengths in fish bile samples obtained after 24 h exposure. A. Emission scans of naphtalene at fixed excitation wavelengths. B. Excitation scans of naphtalene at fixed emission wavelengths. C. Emission scans of phenanthrene at fixed excitation wavelengths. D. Excitation scans of phenanthrene at fixed emission wavelengths. E. Emission scans of pyrene at fixed excitation wavelengths. F. Excitation scans of pyrene at fixed emission wavelengths.

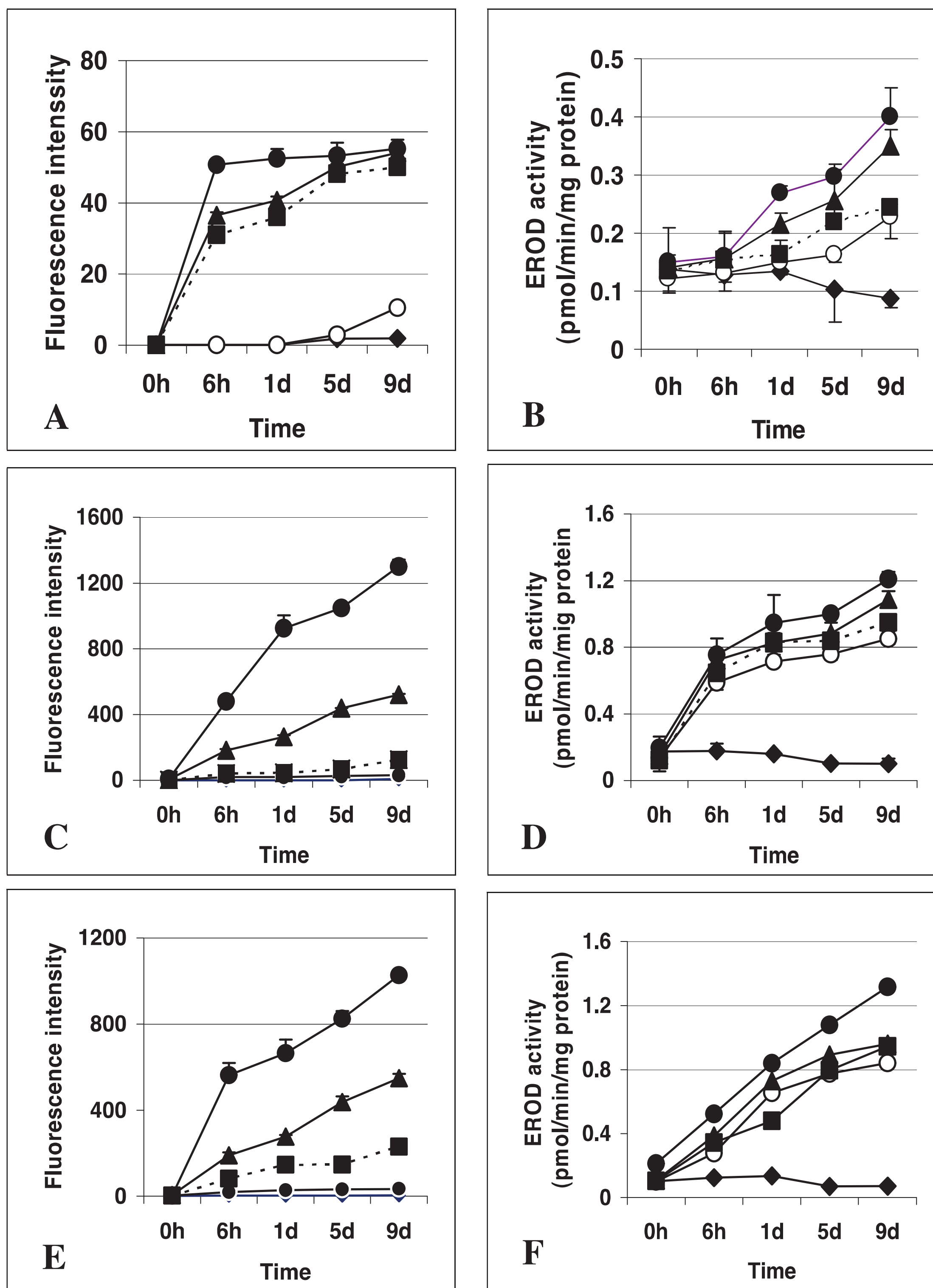

Figure 2. Fluorescence intensities in bile and EROD activities in liver extracts from fish exposed to PAHs for various periods of time (means; S.D.; *n=6*). A. excitation/emission 290/335 nm indicating naphtalene: Control (♦), 0.98 μM (○), 3.9 μM (■), 15.6 μM (▲), 62.5 μM (●). B. EROD activities from the same fishes as in A. C. excitation/emission 260/380 nm indicating phenanthrene: Control (♦), 0.26 μM (○), 1.06 μM (■), 4.21 μM (▲), 16.9 μM (●). D. EROD activities from the same fishes as in C. E. exitation/emission 341/383 nm indicating pyrene Control (♦), 19.8 nM (○), 79.2 nM (■), 312 nM (▲), 1238 nM (●). F. EROD activities from the same fishes as in E.

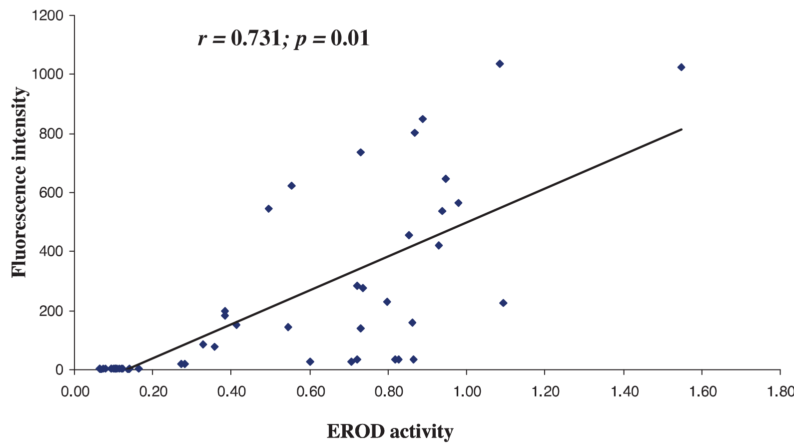

Figure 3. Correlation between flourescense intensity and EROD activity in fish exposed to pyrene.

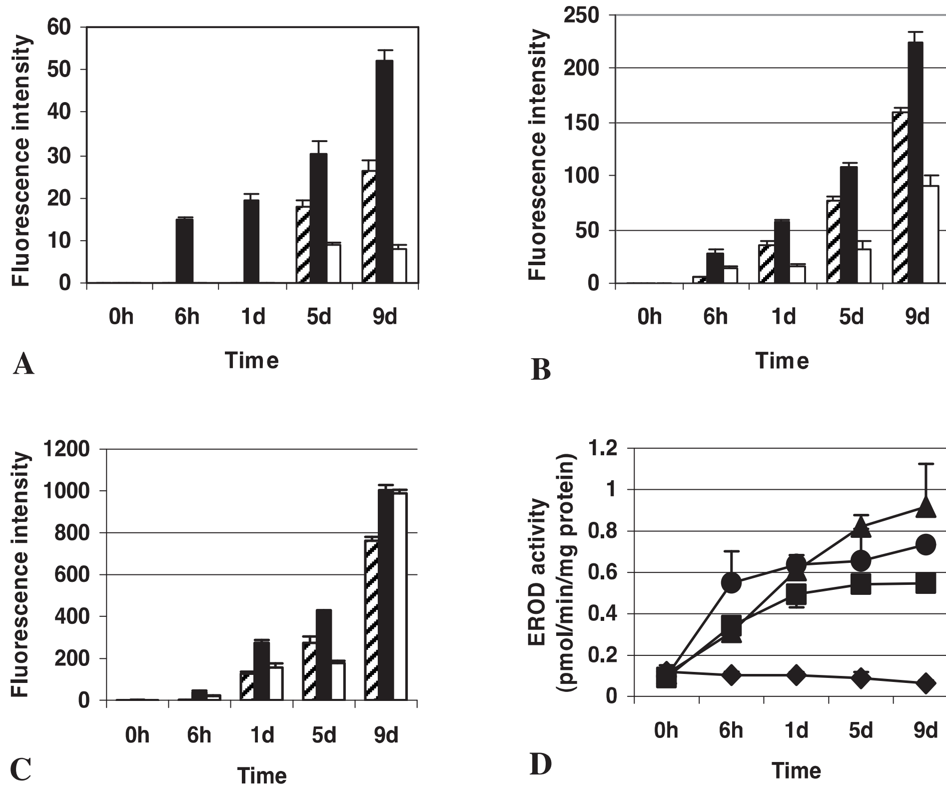

Figure 4. Fluorescence intensities: excitation/emission 290/335 nm (naphtalene; oblique pattern), excitation/emission 341/383 nm (phenanthrene; black bars), excitation/emission 260/380 nm (pyrene; white bars) in bile from: A. fish exposed to lubricating oil; B. fish exposed to gasoline; C. fish exposed to diesel oil. D. EROD activities in liver extracts (means; S.D.; *n=6*) from control fish (♦), and from fish exposed to lubricating oil (■), gasoline (▲), or diesel oil (●).

4. Discussion

Pollution by PAHs is a worldwide problem, which does not show any sign of improvement. Chen *et al*. (2007) who studied the concentration of PAHs in several rivers; found alarmingly high mean values in the water ranging up to 72.4 mg/L (in Minjiang River, China) and in sediment up to 11.0 mg/g (Tianjing River, China).

One important step for dealing with of these conditions lies in providing methods of analysis, which are simple, accurate, fast and inexpensive. Animals in the polluted rivers have, for obvious reasons, been in focus for many such studies. Several investigators have used determination of EROD activity in the various tissues (Aas and Klungsøyr, 1998b; Binelli *et al*., 2006; Camus *et al*., 1998). Fixed wavelength fluorescence (FF) has also provided a valuable tool for PAH quantitation (Lin *et al*., 1996; Camus *et al*., 1998; Escartín and Porte, 1999)

Escartin and Porte (1999) demonstrated that FF techniques could detect fluorescence levels of PAH metabolites with the same accuracy as detected by HPLC-F and GC-MS. However, FF technique is much easier and faster.

Similarly, Lin *et al*. (1996), who compared FF and HPLC-F for the quantitation benzo-a-pyrene and naphthalene metabolites, found a high positive correlation level: $r = 0.89$ and $r = 1.00$, respectively, at the 99% confidence level. This study indicated that due to its convenience, FF techniques could take place of HPLC-F techniques.

Vuorinen *et al*. (2006) used FF techniques and HPLC to study pyrene-type metabolites in the bile from fishes caught in the Baltic Sea. They pointed out that biliverdin and the bile protein could interfere with absorbance measurements at 380 nm. In their experience, however, this interference did not play any major role for biomonitoring of large number of samples.

We conclude that exposure of Nile Tilapia to naphthalene, phenanthrene or pyrene in water, as well as exposure to lubricating oil, gasoline, or diesel oil, results in presence of PAH metabolites in the bile and liver of the exposed fish. With higher concentrations and increased exposure times, the PAH concentrations, as well as the EROD activities in liver extracts, increase. This is in contrast to Gorbi and Regoli (2004) and Camus *et al*. (1998) who found that fluorescence level of PAH metabolites and EROD activity increased after 24 hr exposure but then slowly decreased after 4 days of exposure. This is probably due to fact that their experiments were conducted in the static system.

We conclude that, due to its convenience, simplicity to handle, and cost effectiveness, FF techniques could be used as an accurate, fast and inexpensive tool for measuring of PAH contamination in aquatic ecosystems. The method is relatively uncomplicated and may thus be practical for studies of pollution also in rural areas with limited laboratory facilities.

Acknowledgements

This research work is supported in part by the grant from the Post-Graduate Education, Training and Research Program in Environmental Science, Technology and Management under Higher Education Development Project of the Ministry of University Affairs.

References

Aas E, Beyer J, Goksøyr A. PAH in fish bile detected by fixed wavelength fluorescence. Marine Environment Research 1998a; 46: 225-28.

Aas E, Klungsøyr A. PAH metabolites in bile and EROD activity in North Sea fish. Marine Environment Research 1998b; 46: 229-32.

Binelli A, Ricciardi F, Riva C, Provini A. New evidences for old biomarkers: Effects of several xenobiotics on EROD and AchE activities in Zebra mussel (*Dreissena polymorpha*) Chemosphere 2006; 62: 510-19.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976; 72: 248-54.

Camus L, Aas E, Børseth JF. Ethoxyresorufin-O-deethylase activity and fixed wavelength fluorescence detection of PAHs metabolites in bile in turbot (*Scophthalmus maximus* L.) exposed to a dispersed topped crude oil in a Continuous Flow System. Marine Environment Research 1998; 46: 29-32.

Chen Y, Zhu L, Zhou R. Characterization and distribution of polycyclic aromatic hydrocarbon in surface water and sediment from Qiantang River, China. Journal of hazardous Materials 2007; 141: 148-55.

Escartín E, Porte C. Assessment of PAH pollution in coastal Areas from the NW mediterranean through the analysis of fish bile. Marine Pollution Bulletin 1999; 38: 1200-06.

Finney DJ. Probit Analysis. Cambridge University Press. London 1971.

Gorbi S, Regoli F. Induction of cytochrome P4501A and biliary PAH metabolites in European eel *Anguilla anguill*a: Seasonal, dose- and time-response variability in field and laboratory conditions. Marine Environment Research 2004; 58: 511-15.

Kennedy SW, Jones SP. Simultaneous measurement of cytochrome P450 1A catalytic activity and total protein concentration with a fluorescence plate reader. Analytical Biochemistry 1994; 223: 217-23.

Lin EL, Cormier SM, Torsella JA. Fish biliary polycyclic aromatic hydrocarbon metabolites estimated by fixed-wavelength fluorescence: comparison with HPLC-fluorescent detection. Ecotoxicology and Environment Safety 1996; 35: 16-23.

Shailaja, M.S., R. Rajamanickam and S. Wahidulla: Increased formation of carcinogenic PAH metabolites in fish promoted by nitrite. Environmental Pollution 2006; 143: 174-77.

Vuorinen PJ, Keinänen M, Vuontisjärvi H, Baršienė J, Broeg K, Förlin L, Gercken J, Kopecka J, Köhler A, Parkkonen J, Pempkowiak J, Schiedek D. Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea. Marine Pollution Bulletin 2006; 53: 479-87.

Received 6 February 2010
Accepted 12 April 2010

Correspondence to
Dr. Voravit Cheevaporn
Department of Environmental Science,
Faculty of Science,
Rambhai Barni Rajabhat University,
Chantaburi 22000,
Thailand
Tel: 081-636-4699
Fax: 038-393-491
Email: voravit @buu.ac.th