Efficiency of Methods to Investigate PAHs Exposure in Fish (Nile Tilapia)

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Abstract

The aim of this study was to compare the efficiency of two methods for assessing PAH exposure in fish: measurement of the fluorescence intensity of PAH metabolites in bile (by Fixed Wavelength Fluorescence, FF), and determination of Ethoxyresourfin-O-Deethylase (EROD) activity in fish liver. In addition, we studied oxygen consumption rate and osmoregulation capacity, which are also used as indicators for PAH contamination in water. Nile tilapia (*Oreochromis niloticus*) was exposed to benzene, fluorene, anthracene, chrysene, and benzo[a]pyrene at non-lethal concentrations. The results showed that the fluorescence intensity, EROD activity, and oxygen consumption rate were significantly increased with increased exposure times and PAH concentrations. The maximum values were reached after 16 days' exposure. The measurement of fluorescence intensity of PAH metabolites by FF technique was more sensitive than determination of EROD activity; therefore the FF technique should be preferred in the study of long term exposure. However, the measurement of fluorescence intensity, EROD activity, and oxygen consumption rate can all be used to monitor PAH contamination in water.

Keywords: PAHs; EROD activity; oxygen consumption rate; fixed wavelength fluorescence; Nile tilapia

1. Introduction

PAHs, a group which contains many carcinogenic substances, are widely found in the environment especially in the aquatic environment. They are classified as organic hydrophobic contaminants. They are created and released during the incomplete combustion of organic material, especially fossil fuel, such as oil and coal. The speciation of PAHs in surficial sediments and mussel from the Istanbul Strait and Marmara Sea, Turkey, clearly indicated that the contaminated PAHs originate from high temperature pyrolytic inputs with some slight contribution of petrogenic PAHs (Karacık *et al*., 2008). When PAHs reach to the aquatic environment, they are always adsorbed on solid particles such as suspended particles or bottom sediment because of their low water solubility. Moreover, they can be assimilated into living organisms via the skin which contains lipids.

After absorbtion into the body, some of PAHs such as naphthalene can cause disturbances in the normal physiology: oxygen consumption and change in the activity of respiratory enzymes (Vijayavel and Balasubramanian, 2006). An increase may be observed in malondialdehyde (MDA) levels (Kilanowicz *et al*., 2009) resulting in disruption of endocrine systems (Pollino, 2009). The PAHs are transformed into metabolites with higher water solubility by enzymes in detoxification processes. Most of these metabolites are excreted. However, some PAHs metabolites, such as dial epoxide (metabolite of benzo[a]pyrene), can cause cancer by attaching to the DNA chain and then disturbing DNA translation and transcription.

Due to the effects of PAHs mentioned above, several techniques have been developed to study the fate, the transformation processes, and the physiological and pathological effects of PAHs in the body. These techniques include the measurement of EROD activity and PAHs metabolite fluorescence intensity by the FF method, two methods which are simple, fast, and inexpensive. Pathophysiological changes can be assessed by the measurement of the oxygen consumption rate and the osmoregulation capability.

The aim of this study is to evaluate the efficiency of measuring methods for EROD activity and PAHs metabolite fluorescence intensity in fish exposed to PAHs, affecting factors, and the relationships between the results of the two methods.

2. Methods

The representative organism used was the male Nile tilapia (*Oreochromis niloticus*), its size about 3-4 inches. They were obtained from the Chonburi Inland Fisheries Research and Development Center, Thailand. Before the experiment, they were acclimatized for

about 1-2 weeks in a 1 m^3 tank with aerated tap water and constant temperature at 23.0-25.5^{o}C. Five percent of the water in the tank was replaced every day. The fish was then transferred to the experimental tanks of 30 L, each tank contained 30 fishes. There were seven groups of experimental treatment. The study groups were exposed to benzene concentrations of 0.6, 1.2, 6.4, 12.8 µmol/L (benzene – a monocyclic aromatic compound – was included for comparison with the PAHs). Fluorene and anthracene concentrations were 0.3, 0.6, 3 and 6 µmol/L respectively, and chrysene and benzo[a]pyrene concentrations 0.2, 0.4, 2, 4 µmol/L, respectively. These concentrations were selected based on the subletal concentrations (LC_{50} -96 hr) and No Observed Effect Level (NOEL), while the control group was exposed to 0.2 ml/L acetonitrile which is used as the solvent in other conditions. All treatments were carried out in continuous flow conditions, and 4% of the treated water (including the same aromatic compound concentrations) was replaced every day. The pH, dissolved oxygen concentration (DO), and temperature were measured every day. The fish was then sampled after 0, 2, 4, 8, and 16 days for measurement of oxygen consumption rate and osmoregulation capability.

Fish bile metabolites were extracted by using a method modified from Aas *et al*. (1998) and Britvic *et al*. (1993). Briefly, the fish samples were soaked in ice and then its bile was withdrawn and filled in microcentrifuge tubes. Next, 48% ethanol was be added to each microcentrifuge tube in a ratio of 1:2,500. This process was carried out at a constant temperature of about 4°C for preventing enzyme activities. Using a UV/VIS spectrophotometer (UNICAM) at wavelengths Ex 530/Em 590 nm we measured the luminescence levels of PAH metabolites. Fish liver was extracted for determination of EROD activity following IPCS (1998) and Burke and Mayer (1974). The total protein analysis was studied in 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 calculate a standard curve for the measurements of protein concentration in the samples (Bradford, 1976). For the statistical analysis, the variance was analyzed by ANOVA, and the normal of data testing was analyzed by the Shapiro-Wilk test and log-transformed. SPSS analysis was carried out for estimating relations between the fluorescence intensity of the aromatic hydrocarbon metabolites in bile and EROD activity level in liver at a confidential level of 95%.

For physiological studies, fish were put in a respiration chamber, which was filled with pasteurized, aerated water. After 24 h of acclimatization under constant temperature at 25°C, this chamber was closed. Oxygen consumption was measured using a closed system respirometer (volume, 750 ml). An oxygen electrode (1302 oxygen electrode) was fitted airtight through the lid of the respiration chamber and connected to the probe holder of the oxygen meter (Strathkelvin Instruments oxygen meter model 781). After 10 minutes of equilibration, the oxygen pressure (PO_2) was recorded every 10 min (the first value is the initial PO_2) for 30 min. Finally, the fish was weighed and then the oxygen consumption rate was calculated by using the equation shown below.

$$MO_2 = (PO_2\,start - PO_2\,end)\;x\;a\;x\;V\;x\;60/t/W\;(µmol/g.h)$$

where a = water solubility of oxygen at that temperature (µmol/L.torr)

V = water volume in chamber (L)
t   = experimental time period (h)
W = body weight of fish (g)

Blood of Nile tilapia was withdrawn from the blood vessel inside of caudal skeleton for further measurement of blood osmolarity using an osmometer (Wescos Vapour pressure osmometer model 5520).

For protein determination, we used a method modified from ICES (1998) and Burke and Mayer (1974). In brief, the liver sample was placed in a microcentrifuge tube filled with the homogenizing buffer (4°C) at a ratio of 1/5-1/10 g/ml (w/v). Then, it was homogenized by a homogenizer at 700 rpm and centrifuged at 10,000 rpm (4°C) for 20 min. Finally, the supernatant (S9) was withdrawn for measuring EROD activity level and protein concentration.

3. Results and Discussion

3.1. Oxygen consumption and osmoregulation

The results show that an increase in both exposure time and PAHs or benzene concentrations resulted in increases in both the oxygen consumption rate and osmoregulation capacity. For oxygen consumption, the maximum rate was reached after 16 days (4 µmol/L), as shown in Fig. 1.

The oxygen consumption rate and osmoregulation were increased (Fig.1), possibly to serve the elevated detoxification activities which were induced by PAHs exposure (4 µmol/L).

There was an increase of the fluorescence intensities of the metabolites of benzene, fluorene, anthracene,
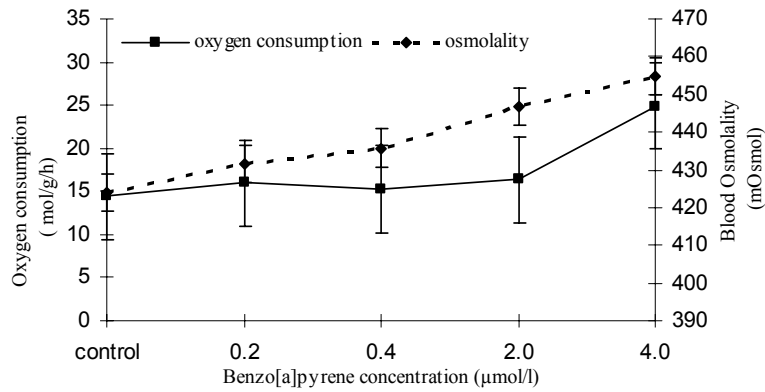
Figure 1. The oxygen consumption rate and osmoregulation capability in Nile Tilapia exposed for 16 days to benzo[a]pyrene (means±S.D.; *n=10*).
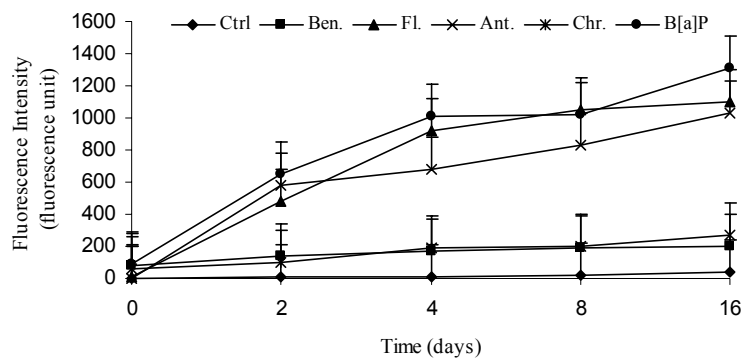

Figure 2. Fluorescence intensity of metabolites of benzene, fluorene, anthracene, chrysene, and benzo[a]pyrene in fish bile. (means±S.D.; *n=5*).
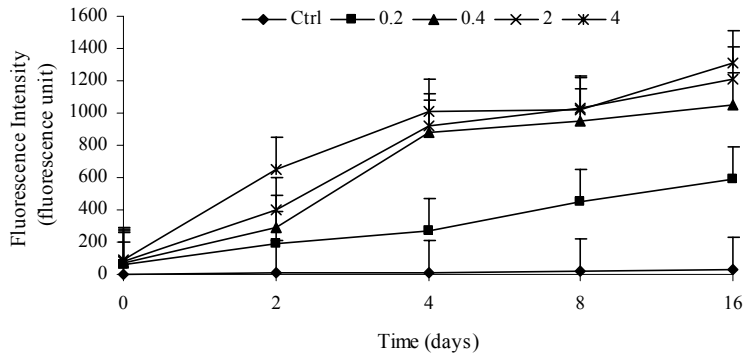

Figure 3. Fluorescence intensity of benzo[a]pyrene metabolite (means±S.D.; *n=5*).

chrysene, and benzo[a]pyrene in fish bile after the fish had been exposed to 4 µmol/L of the substances (Fig. 2). The increase was augmented by increasing the exposure time, and the highest intensity, reached at 16 d, indicates a significant increase.

The fluorescence intensity of B[a]P metabolite increased significantly with increasing concentrations of B[a]P concentrations (Fig. 3). The highest fluorescence intensity was reached at the concentration of 4 µmol/L.

An increase of fluorescence intensity of PAHs metabolites with increasing exposure times and concentrations was caused by the biotransformation in the detoxifying processes in the fish. When the fish had been exposed to PAHs, these were transformed to metabolites by the CYP1A enzyme in the detoxification process. Thus, the amounts PAHs metabolite (reflected by the fluorescence intensity) were increased with exposure time and PAHs concentration. Yang and Baumann (2005), Aas and Goksøyr (1998), and Aas *et al.* (2000)
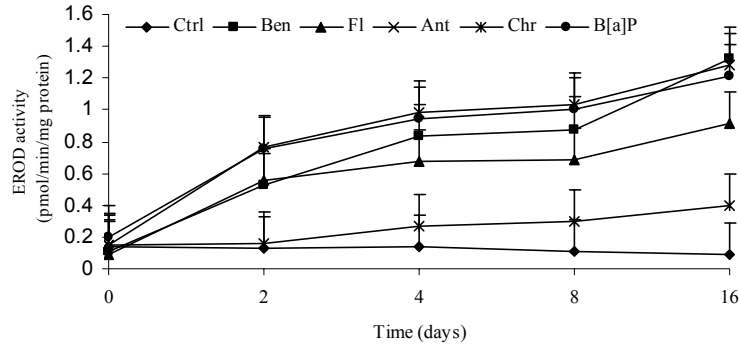
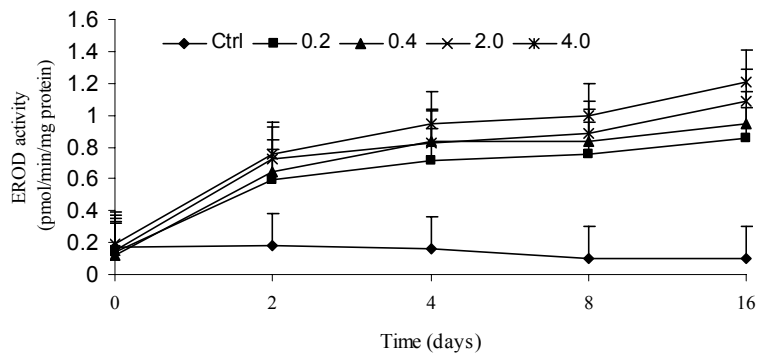Figure 4. EROD activity in fish exposed to PAHs (means± S.D.; *n=5*).

Figure 5. Increase in EROD activity with increasing benzo[a]pyrene concentrations (means±S.D.; *n=5*).
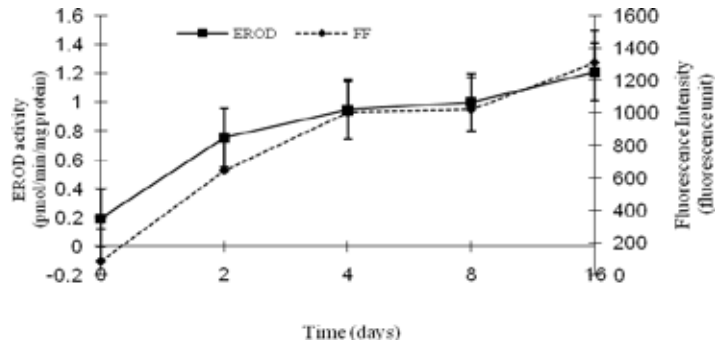

Figure 6. Fluorescence intensity and EROD activity relationship against time exposure (means±S.D.; *n=5*).

have previously reported similar results.

EROD activity in exposed fish was increased with exposure times and PAHs concentrations (Figs. 4-5). EROD activity reflects the amount of CYP1A enzyme, which thus was significantly increased when fish was exposed to the inducer, in this case benzo[a]pyrene.

For the relationship between PAHs metabolites fluorescence intensity and EROD activity, the results indicate that there was a statistically significant positive correlation between the fluorescence intensity of PAHs

metabolites in fish bile and EROD activity in liver (Figs. 6-7).

As stated above, the EROD activity is measuring the amount of CYP1A enzyme involved in phase I of detoxification (transformation), and the fluorescence intensity reflects the amount of PAHs and benzene metabolites, the product from PAHs transformation process. Fenet *et al*. (2005) found that bile PAHs metabolites in EROD activity in caged fish collected from downstream near to local industrial effluent outlets were
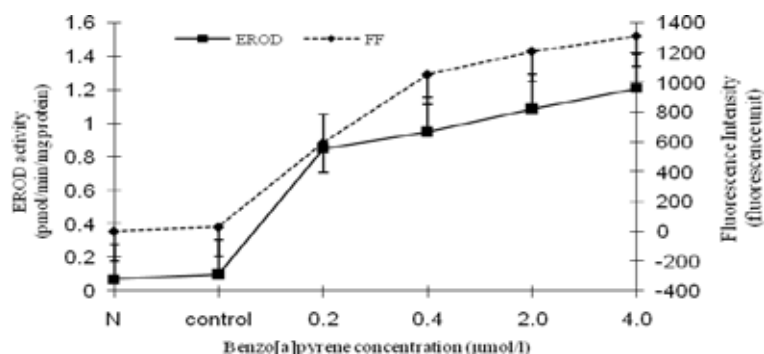
Figure 7. Fluorescence intensity and EROD activity relationship in fish exposed to various B[a]P concentrations (means±S.D.; *n=5*).

increased after 14 days of caging. In addition, Jönsson (2009) studied the induction of EROD activity and CYP1A as well as the adduction formation caused by PAH in Zebrafish (*Danio rerio*). They found that these can be used as kinetic factors for evaluating the effect of PAH. We conclude that the measured values were significantly positively correlated and increased with exposure time and PAHs concentrations.

4. Conclusion

When fish is exposed to PAHs, a group of toxicants, its physiological and detoxification responses are induced. Thus, the oxygen consumption rate and osmoregulation are increased, presumably to serve the elevated detoxification activities. For detoxification, CYP1A enzyme levels are increased to allow a higher rate of detoxification. This enzyme transforms parent PAHs to PAHs metabolites with higher water solubility, which are more readily excreted. By this relationship, we can use the measurement of produced CYP1A (EROD activity) and the fluorescence intensity of PAHs metabolites in assessment and monitoring the pollution condition in the water reserves. Moreover, both these methods are practical to apply in environmental studies, being both simple, fast, and inexpensive.

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