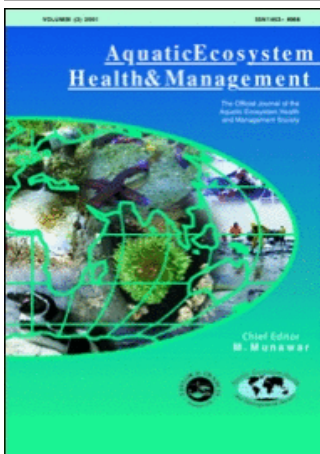


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Induction of ethoxyresorufin-O-deethylase (EROD) activity in the liver of black porgy (*Acanthopagrus schlegelii*) exposed to benzo(a)pyrene

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Induction of ethoxyresorufin-O-deethylase (EROD) activity in the liver of black porgy (*Acanthopagrus schlegeli*) exposed to benzo(a)pyrene

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This paper studied the induction kinetics of the enzyme 7-ethoxyresorufin O-deethylase in the liver of the marine fish, Acanthopagrus schlegeli following the exposure to benzo (a)pyrene for up to 14 days at concentrations of 0.5, 1.0, 2.0, 5.0 $\mu\text{g l}^{-1}$. The results demonstrated a dose-response relationship between hepatic enzyme activities induction and benzo(a)pyrene concentration. Hepatic enzyme activities were significantly induced by the second day with exposure to the lower concentrations of benzo(a)pyrene (0.5 $\mu\text{g l}^{-1}$ and 1.0 $\mu\text{g l}^{-1}$), and at 12 hours of exposure for the higher concentrations (2.0 $\mu\text{g l}^{-1}$ and 5.0 $\mu\text{g l}^{-1}$). After 7 days depuration, the activities of the enzyme of fish returned to the control level. This indicated that while benzo(a)pyrene has an effect on the hepatic enzyme activity, the fish can eliminate a major part of BaP metabolites.

Keywords: PAH, depuration experiment, kinetics process

Introduction

Benzo(a)pyrene (BaP), an unsubstituted polyaromatic hydrocarbon (PAH), is widely dispersed in the marine environment (Leonard and Hellou, 2001). It can be accumulated through different processes, for example, by direct uptake from water by gills or skin, and consumption of contaminated food (Livingstone, 1993; Lopez-Barea, 1995; Lopez-Barea and Pueyo, 1998; MacRae et al., 1998). Generally, fish have a well-developed mixed function oxidize (MFO) system that can rapidly metabolize BaP into hydrophilic products that are more easily excreted (Britviç et al., 1993; Aas et al., 1998; Gagnon and Holdway, 2000). Nevertheless, depending on the chemical structure and

level of exposure, BaP and its metabolites are potentially toxic, as well as mutagenic and/or carcinogenic effects in fish and other vertebrates including humans (MacRae and Hall, 1998; Monteiro et al., 2000).

Benzo(a)pyrene has been shown to induce marked metabolic activity. A well-characterized biochemical marker in fish and other vertebrates is the induction of MFOs or monooxygenases dependent on hepatic cytochrome P4501A (CYP1A1). Contaminants such as PAH induce liver CYP1A1 in fish, so it is used as a biomarker of exposure. Enzyme activity associated with CYP1A1 has been determined using 7-ethoxyresorufin as a substrate. The measurement of the activity of 7-ethoxyresorufin O-deethylase (EROD)

is the most widely used catalytic probe for determining the induction response of CYP1A1 in fish (Lemaire-Gony and Lemaire, 1992; Lemaire et al., 1996; Pacheco and Santos, 1997, 1998, 1999; Goksøyr and Husøy, 1998; Jaksic et al., 1998). It is known that EROD activities are easily induced by xenobiotic compounds including PAHs and numerous studies have demonstrated the induction of liver EROD activities in different fish species exposed to BaP (Raza et al., 1995; Fenet et al., 1998; Gagnon and Holdway, 2000; Livingstone et al., 2000).

The Black Porgy (*Acanthopagrus schlegelii*) is an important economic fish species in the South China Sea. It is a shallow-sea warm-water bottom-dwelling species and inhabits the sandy and rock zone. This sentinel species is resistant to fluctuations in temperature, salinity, food availability, and it grows rapidly. A few studies using the Black Porgy as an experimental specimen have been reported (Haasch et al., 1993). The objective of this paper is to study the induction kinetics of EROD activities with exposure to benzo(a)pyrene, and to investigate the response relationship between the induction of hepatic EROD activities in fish and BaP concentrations.

Materials and methods

Chemicals

Benzo(a)pyrene, reduced β -nicotinamide adenosine-diphosphate (NADPH), 7-ethoxyresorufin and resorufin were purchased from Sigma chemical, UK. All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Experiment design and homogenate preparation

Black Porgy, with an average weight of 11.2 ± 1.5 g and average length of 9.12 ± 1.3 cm, were collected from Liuwudian sea fish farm, in Tong'an bay. The fishes were transported to the laboratory and placed in a pond at 22°C for 1 wk for acclimatization prior to the start of the experiment. The water used for all the experiments was sand-filtered seawater. During the experiment, the water was constantly aerated (dissolved oxygen 7.8–8.2 mg l⁻¹; pH 7.8–8.2; salinity 35‰; temperature 22°C). The fish were fed on shrimp every two days in the acclimation period, but food was not provided throughout the experimental period. Sea water was changed once a day.

Polyethylene boxes (63 × 115 × 50 cm³) were used as an experimental container. The BaP was dissolved in acetone, and preserved avoiding light. More than 300 fishes were divided randomly into 10 groups and two groups were used for each concentration of BaP (0.5, 1.0, 2.0, 5.0 $\mu\text{g l}^{-1}$) plus one blank control group and one acetone group. The samples were collected at the exposure time of 2 h, 6 h, 12 h, 1 d, 2 d, 4 d, 7 d and 14 d. After the exposure treatment, residual fishes were depurated in sand-filtered seawater for 1 wk.

Liver removed from each sacrificed fish was immediately weighed and stored first in liquid nitrogen at -80°C. The above procedures were completed within a short time (<2min, for each fish) to prevent loss of enzyme activity. A 300 ± 15 mg slice of liver was homogenized in 1.5 ml phosphate buffer (0.1 M, pH 7.6) by an automatic homogenizer, then centrifuged at 10,000 g for 20 min at 4°C. Supernatant liquids were kept at 4°C and assayed within 1 h.

Determination of EROD activity and BaP concentration in water

The EROD activity was assayed fluorometrically by measuring the rate of enzymatic deethylation of 7-ethoxyresorufin to resorufin over 8 min at excitation and emission wavelengths of 530 nm and 590 nm, respectively, on a Cary eclipse fluorescence spectrometer. The EROD was measured using a slight modification of the methods described by Hodson et al. (1996) and Kirby et al. (1999). All assay reagents were kept in a water bath, at 20°C. The reaction mixture contained 3.9 ml assay buffer, 40 μl liver homogenate, 20 μl 0.40 mmol ethoxyresorufin substrate and 20 μl 5 μmol resorufin as the internal standard. The standard equates to an addition of 250 pmol of resorufin against which the assay was calibrated. To ensure quality control, assay substrate and standards were made up in bulk batches and 1 to 2 ml aliquots were frozen. Fresh aliquots were defrosted immediately prior to the assay. The reaction was initiated by the addition of 20 μl 0.25 mmol NADPH and the emission reading was recorded from 0 to 8 min at half-min intervals. The EROD activity was normalized to protein content and expressed as pmol resorufin min⁻¹ mg⁻¹ protein. Protein analyses were carried out on the same liver homogenate as the EROD activity measurements using a plate reader modification of the Bradford method with a bovine serum albumin standard. The concentrations of BaP in water were determined according to the literature (Maskaoui et al., 2002).

Table 1. Concentrations of BaP ($\mu\text{g l}^{-1}$) in experiment water of different exposure groups.

Groups/ time	2 h	12 h	1 d	2 d	4 d	7 d	14 d	21 d
Blank control	0.0001	ND	0.0001	ND	ND	ND	0.0001	ND
Acetone control	ND	ND	ND	ND	0.0001	ND	ND	0.0001
Bap ($0.5 \mu\text{g l}^{-1}$)	0.463	0.4844	0.4672	0.4865	0.4779	0.4926	0.4515	ND
Bap ($1.0 \mu\text{g l}^{-1}$)	1.0406	1.0378	1.0371	1.0329	1.0338	1.0403	1.0365	0.0003
Bap ($2.0 \mu\text{g l}^{-1}$)	2.0423	2.0276	2.0374	2.0351	2.0362	2.0298	1.9761	0.0003
Bap ($5.0 \mu\text{g l}^{-1}$)	4.6786	4.4589	4.8275	4.4210	4.7250	4.5584	4.7024	0.0002

ND: not determined.

Statistical analysis

Results were reported as mean \pm SD. The data was processed by parametric statistical analysis using sigma plot software followed by the t-test. $P < 0.05$ was accepted as significant, $P < 0.01$ was accepted as strongly significant.

Results

The concentrations of BaP in experiment water in different exposure groups are listed in Table 1. The results showed that no significant differences were observed in concentrations of BaP between experiment water and design concentrations.

The responses of EROD to different concentrations of BaP and different exposure times are shown in Figure 1. During the exposure, hepatic EROD activity had

no significant alteration either in blank control group or in acetone group. In the BaP group at the lowest concentration, $0.5 \mu\text{g l}^{-1}$, EROD activities were significantly different in the early two days' exposure, reached the highest value at the second day and showed the most significant difference at that time. It was found that the activity decreased somewhat after 4 or 7 d exposure but still showed a significant difference from the blank control. The activities of EROD tended to increase at the beginning and then decrease, and then increase again at the concentrations of 1.0, 2.0, and $5.0 \mu\text{g l}^{-1}$ BaP. The activity of EROD was lowest in the groups at $1.0 \mu\text{g l}^{-1}$ BaP after 1 d exposure and then increased to the highest value after 2 d exposure. The activity of EROD reached its highest value after 2 h exposure to $2.0 \mu\text{g l}^{-1}$ BaP but dropped to its lowest value with 2 d exposure to this concentration. The activity of EROD reached its highest value after 2 h exposure to $5.0 \mu\text{g l}^{-1}$ BaP,

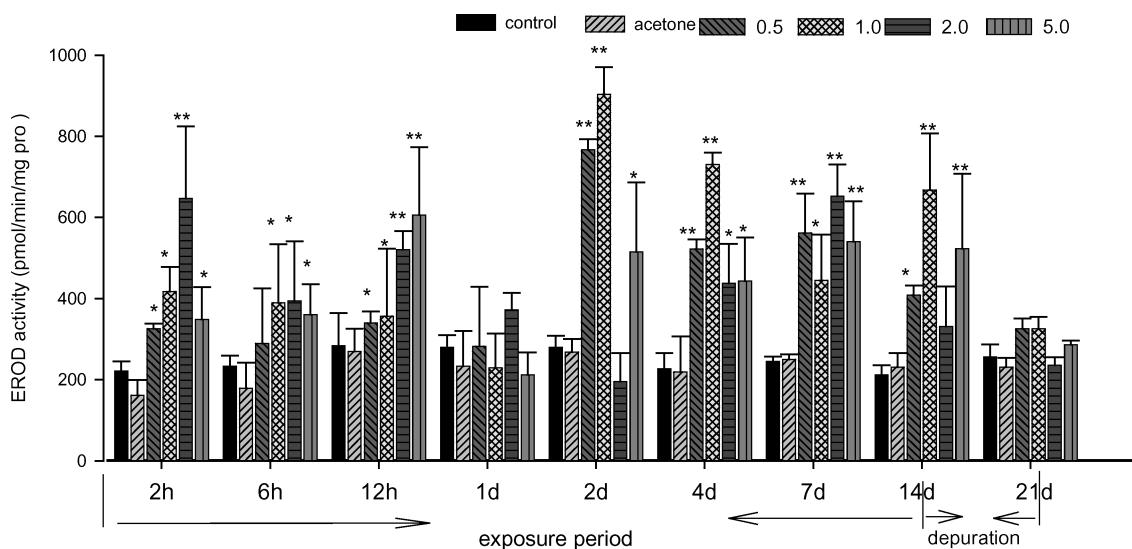


Figure 1. Induction kinetics of hepatic EROD activity ($\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$) of *Acanthopagrus schlegelii* after 2 h, 6 h, 12 h, 1 d, 2 d, 4 d, 7 d and 14 d exposure to 0, 0.5, 1.0, 2.0 and $5.0 \mu\text{g l}^{-1}$ benzo(a)pyrene and depurated in sandy-filtered seawater for 7 d after the exposure experiments, respectively. Values represent mean \pm SD. Difference from control: (* $P < 0.05$, ** $P < 0.01$).

but the activity decreased to its lowest value after 1 d exposure.

Hepatic EROD activities tend to be induced with the elevation of the exposure level with 12 h exposure; exposure to $2.0 \mu\text{g l}^{-1}$ BaP was found to have a significant induction effect after 1 d exposure in contrast with the activity of EROD in the other groups. Exposure to 0.5, 1.0 and $5.0 \mu\text{g l}^{-1}$ BaP induced activity of hepatic EROD; exposure at $0.5 \mu\text{g}$ and $1.0 \mu\text{g l}^{-1}$ BaP manifested the most significant induction. However, inhibition was found in fishes exposed to $2 \mu\text{g l}^{-1}$ BaP. After 4, 7 or 14 d exposure, BaP induced the activity of EROD, but there was no significant difference in the induction of EROD between different exposure levels.

After 7 d depuration, hepatic EROD activities decreased in all exposure levels, with no statistical differences compared with the blank control group.

Discussion

The concentrations of BaP in seawater from Xiamen Western Sea were reported to range from 0.92 to $3.32 \mu\text{g l}^{-1}$ (Maskaoui et al., 2002). Therefore, the kinetics of the hepatic EROD activities in Black Porgy exposed to the concentrations of 0.5, 1.0, 2.0, $5.0 \mu\text{g l}^{-1}$ BaP for 2h, 6h, 12h, 1d, 2d, 4d, 7d and 14d were studied in this experiment.

Experimental results demonstrated that the strong significant induction of EROD activity exposure to BaP at the concentration of $5.0 \mu\text{g l}^{-1}$ seemed to be earlier than that of exposure to BaP at the concentrations of 0.5 and $1.0 \mu\text{g l}^{-1}$. The result of EROD activities in *Mugil so-iuy* injected with $50 \mu\text{g l}^{-1}$ BaP (Wang et al., 2003) showed that the strong significant induction of EROD activity seemed to be earlier than that with an injection to $5 \mu\text{g l}^{-1}$, similar to our experimental results. Moreover, this response indicated that Black Porgy was sensitive to BaP and therefore suitable for monitoring pollution by this chemical.

Our study found a trend, that is, an increase at the beginning of exposure followed by a decrease, then another increase in EROD activities changes in the liver of Black Porgy exposed to the concentrations of 1.0, 2.0, $5.0 \mu\text{g l}^{-1}$ BaP. A possible reason is that when exposed to BaP, the hepatic EROD was immediately induced, but the induction of EROD activities decreases with continued exposure to the high concentrations of BaP, which we assume to relate to the lipid peroxidation in the liver. Previous studies (Hendricks et al., 1985; Devaux et al., 1998; Pacheco et al., 1998) had shown that there are more than twenty kinds of metabolic processes that can cause lipid peroxidation in the liver during the pro-

cesses of BaP metabolism. Ultimately, they can lead to CYP1A1, so that the EROD activities decrease. Au et al. (1995) observed a good correlation between the hepatic EROD activities in *Solea ovata* and the concentrations of BaP. Consistent with these results, our study showed that at the beginning of the exposure (2, 6, and 12h), the hepatic EROD activities increased with the elevated concentration of BaP exposure.

Many field and laboratory experiments showed that the activities of hepatic EROD are induced by PAHs and polychlorinated biphenyls (Hawkins et al., 1988; Lemaire et al., 1992; Raza et al., 1995; Livingstone et al., 2000; Holdway et al., 2000). The field studies on the leaping mullet (*Liza saliens*) and common sole (*Solea vulgaris*) showed that there was a very good relationship between hepatic EROD activities and the concentrations of PAHs in the water (Arinç and Sen 1999; Gravato and Santos, 2002). Gagnon and Holdway (2002) determined the activities of hepatic EROD activities in the sand flathead (*Platycephalus bassensis*) in the Phillips Bay of Australia and found that the activities of EROD in the industrial and sea route areas were much higher than that in other less traveled site. Hepatic EROD activities in Catfish (*Italurus punctatus*) exposed to the water polluted by PAHs and PCBs was 4, 3, and 5 times higher than that of controls after 2, 4 and 6 wk respectively (Van der Oost et al., 1996).

After 7 d depuration, the activity of hepatic EROD returned to the control level. On the one hand, this demonstrates the fish possesses a high capability to eliminate a major part of BaP metabolites. On the other hand, the MFO system has strong resilience. This result is in good accordance with the observations by Britvić et al. (1993), who recorded BaP MFO activity at background levels 15 d after the end of exposure.

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