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Relationship between metabolic enzyme activities and bioaccumulation kinetics of PAHs in zebrafish (*Danio rerio*)

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ABSTRACT

Many studies have investigated bioaccumulation and metabolism of polycyclic aromatic hydrocarbons (PAHs) in aquatic organisms. However, lack of studies investigated both processes simultaneously, and the interaction between these two processes is less understood so far. This study investigated the bioaccumulation kinetics of PAHs and metabolic enzyme activities, including total cytochrome P450 (CYPs) and total superoxide dismutase (T-SOD), in zebrafish. Mature zebrafish were exposed to the mixture of phenanthrene and anthracene under constant concentration maintained by passive dosing systems for 16 days. The results showed that PAH concentrations in zebrafish experienced a peak value after exposure for 1.5 days, and then decreased gradually. The bioaccumulation equilibrium was achieved after exposure for 12 days. Both of the uptake rate constants (k_u) and the elimination rate constants (k_e) decreased after the peak value. The variation of PAH concentrations and metabolic enzyme activities in zebrafish had an interactive relationship. The activities of CYPs and T-SOD increased initially with the increase of PAH concentrations, but decreased to the lowest state when PAH concentrations reached the peak value. When the bioaccumulation equilibrium of PAHs was achieved, CYPs and T-SOD activities also reached the steady state. In general, CYPs and T-SOD activities were activated after exposure to PAHs. The decrease of PAH concentrations in zebrafish after the peak value may be attributed to the great drop of k_u and the variation of CYPs activities. This study suggests that an interactive relationship exists between bioaccumulation kinetics of PAHs and metabolic enzyme activities in aquatic organisms.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic contaminants in the environment, and it has been detected in many rivers around the world (Dong et al., 2015; Kanzari et al., 2014; Rabodonirina et al., 2015; Xia et al., 2009; Yu et al., 2016). PAHs derived from both natural and anthropogenic sources are generally produced by incomplete combustion of carbon, petrochemical production, and cement manufacture (Dudhagara et al., 2016; Patrolecco et al., 2010). Urban runoff, industrial and municipal sewage discharge, and atmospheric precipitation are all routes making PAHs enter the natural water body. PAHs have been listed as priority pollutants by US Environmental Protection Agency (US EPA) due to its environmental concerns and health risks.

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PAHs are a typical class of hydrophobic organic compounds (HOCs) with properties of low water solubility, high octanol–water partition coefficients (k_{ow}), and hydrophobicity, and characteristics of persistence, toxicity, carcinogenicity, and mutagenicity in the environment. PAHs have been detected in invertebrates, fish, and many other aquatic organisms (Boscolo et al., 2007; Dsikowitzky et al., 2011; Wang et al., 2016). Aquatic organisms, such like fishes, can take up PAHs through gills by passive uptake from water and ingestion of contaminated sediments or other aquatic organisms in lower trophic levels. Due to the properties of hydrophobicity and lipophilic, PAHs may tend to be accumulated by organisms. Considering the characteristics of toxicity, PAHs have drawn public and society concerns because they can be accumulated in human through food web, and pose acute and chronic toxicity on human health.

In order to reduce the harm of PAHs to the environment and human beings, many studies have been carried out to investigate its bioaccumulation and biotransformation process in aquatic organisms (Wang et al., 2011). For bioaccumulation, researchers always study its equilibrium and kinetics. Bioaccumulation factor (BAF) is always used to show the ability of PAHs of interest to be accumulated in organisms when bioaccumulation equilibrium is reached. Organisms have higher concentrations of PAHs with higher BAF values when concentrations of PAHs in the waterbody remain the same. When it comes to bioaccumulation kinetics, two compartment dynamic model described by two key parameters of the uptake rate constant (k_u) and the elimination rate constant (k_e) is generally used to study the bioaccumulation process in organisms (Landrum, 1989). For biotransformation, metabolic process, metabolites, and metabolic enzyme activities are important fields focused by many researchers. To date, it has been reported that PAHs in aquatic organisms can be metabolized by cytochrome P450, and bio-transformed into hydrophilic derivatives through phase 1 and phase 2 metabolism (Dong et al., 2009; Matsuo et al., 2006). The planar structures make PAHs have affinity to interact with aryl hydrocarbon receptor (AHR), which can stimulate the expression of a number of detoxifying enzymes including cytochrome P450 to convert PAHs to water soluble derivatives (Billiard et al., 2006; Incardona et al., 2006; Timme-Laragy et al., 2007). Along with metabolic process of PAHs, reactive oxygen species (ROS) will be produced as byproducts, which can pose oxidative stress to organisms (Orbea et al., 2002). As the main antioxidant enzymes comprising the cell defensive system, superoxide dismutase is a very significant and universe enzyme eliminating excess ROS (Liu et al., 2015).

However, lack of studies investigated bioaccumulation and biotransformation simultaneously. Since bioaccumulation is a dynamic process, biotransformation process will have influence on bioaccumulation. Once PAHs enter the organisms, they will be bio-transformed into metabolites by enzymes. Therefore, metabolic process can have influence on the elimination process of PAHs, which can further affect the bioaccumulation kinetics of PAHs in organisms. But the interaction between bioaccumulation kinetics of PAHs and metabolic enzyme activities is less understood so far.

In the present study, phenanthrene and anthracene were studied. These two kinds of PAHs represent three-ring PAHs with logarithmic octanol–water partition coefficients ($\log k_{ow}$) of 4.57 and 4.54 for phenanthrene and anthracene, respectively (Lai et al., 2015). Zebrafish (*Danio rerio*) was chosen as the tested organism because it is a model aquatic organism and has been used in many scientific researches. It has been reported that approximately 70% human genome sequence was orthologous to zebrafish reference genome sequence (Howe et al., 2013), which led to the widespread use of zebrafish in studying the effect of pollutants on human health. The activities of total superoxide dismutase (T-SOD) and total cytochrome P450 (CYPs) in zebrafish were measured. The passive doing system, which can be regarded as an infinite source of PAHs in the aqueous phase (Birch et al., 2010; Gouliarmou et al., 2012; Smith et al., 2010), was applied to keep the freely dissolved concentrations of PAHs constant during the exposure. The aims of this study were to (1) investigate the bioaccumulation kinetics of two PAHs; (2) examine the activities of metabolic enzymes under exposure to PAHs; and (3) investigate the relationship between metabolic enzyme activities and bioaccumulation kinetics of PAHs.

1. Material and methods

1.1. Chemicals

Phenanthrene and anthracene in solid phase were purchased from the Johnson Matthey Company (Alfa Aesar, USA), with purity >98%. The standard solution mixture of PAHs at a certified concentration of 200.0 μg/mL for each PAH in dichloromethane and methanol solution (1:1, V/V) was purchased from AccuStandard, USA. The internal standard substance *m*-terphenyl was purchased from AccuStandard with purity >98%. The poly-(dimethylsiloxane) (PDMS) elastomer was prepared from a Silastic MDX4-4210 BioMedical grade Elastomer kit (DOW Corning) purchased from Baili (Shanghai) Medicinal Materials Trade Inc. of China. The reagent kits for protein, CYPs, and T-SOD assay were purchased from Nanjing Jiancheng Bioengineering Institute, China. High-performance liquid chromatography grade methanol, dichloromethane, hexane, and acetone were purchased from J.T. Baker (USA); all other analytical-grade reagents and chemicals were purchased from Beijing Chemical Reagents, China. Milli-Q water (Super Q-treated, Millipore, USA) was used in the present study.

1.2. Establishment of the passive dosing system

Passive dosing dishes were 60 mm diameters and a total of 12 g mixture of PDMS prepolymer with the matched catalyst (10:1, *m/m*) were added to each dish. These dishes were vacuumed to eliminate trapped air, and then placed at room temperature for 24 hr, and subsequently placed in an oven at 110°C for 48 hr to complete curing. Cured dishes were immersed in methanol for at least 72 hr to remove impurities and oligomers. In order to get the specific concentrations of PAHs maintained in the water through passive dosing dishes, the partition coefficients between methanol loading solutions (MeOH) and artificial freshwater (AFW, $K_{MeOH:AFW}$) should be obtained in the first place. The passive dosing system was established through the loading procedure (partition between MeOH and cured dishes) and the dosing procedure (partition between cured dishes and water). The detail information on the

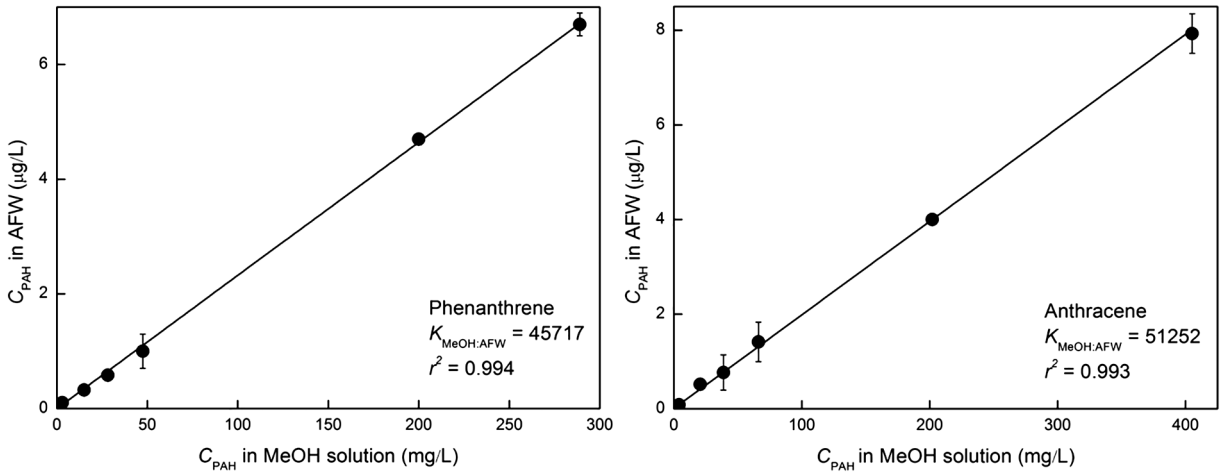

Fig. 1 – The relationship between PAH concentrations in MeOH and AFW. PAH: polycyclic aromatic hydrocarbons; AFW: artificial freshwater.

experimental operation and calculation of the $K_{MeOH:AFW}$ has been described clearly in our previous study (Xia et al., 2015).

According to the results shown in Fig. 1, the $K_{MeOH:AFW}$ values for phenanthrene and anthracene were 4.57×10^4 and 5.13×10^4, respectively. Based on $K_{MeOH:AFW}$ values, the concentrations of PAHs in MeOH can be calculated to make PAH concentrations in AFW maintain at 10 μg/L. Thus the MeOH was prepared by dissolving 0.4572 g of phenanthrene and 0.5125 g of anthracene per liter of methanol. After loading, these dishes were rinsed by Milli-Q water for the bioaccumulation experiment. Two passive dosing dishes were placed in each tank with 1.5 L water at least 24 hr before the start of exposure, which was long enough for PAHs to reach equilibrium between PDMS and AFW.

1.3. Bioaccumulation experiment

Mature zebrafish of similar size and the same generation were tamed in AFW for at least one week in our laboratory. They were cultured at (23 ± 0.5)°C during day and night under a 14 hr:10 hr (light:dark) photoperiod and fed for commercial fodder daily. The fish were placed in AFW without feeding for at least one day to clear the gut before the start of exposure.

The bioaccumulation experiments were conducted in 15 cm × 10 cm × 20 cm glass tanks with 1.5 L AFW. A total of 22 tamed zebrafish were placed in each tank. The concentration of each PAH in each tank was maintained at 10 μg/L by the passive dosing system. The zebrafish were also cultured at (23 ± 0.5)°C during day and night under a 14 hr:10 hr (light:dark) photoperiod, but fed for nothing to avoid the influence of predation. To compensate the evaporation loss of water, sterilized deionized water with 10 μg/L PAH was added to each tank every day to keep the total volume constant at 1.5 L and ionic strength constant. Zebrafish were sampled after exposure for 0, 0.5, 1, 1.5, 2, 3, 5, 7, 9, 12, and 16 days. At each time point, two fish were transferred from each tank by fish net to a glass beaker and rinsed with Milli-Q water. After that, the heads of zebrafish were cut down and then they were dried with filter paper and placed on aluminum foils to obtain the wet weight. The bioaccumulation experiment was designed in sixtuple with the same concentration of each PAHs at 10 μg/L during the exposure. Sampled zebrafish in triplicate were used to study the bioaccumulation kinetics. The sampled zebrafish with the wet weight obtained were stored at −20°C until subsequent procedure. The other sampled zebrafish in triplicate were set to investigate the activities of metabolic enzymes during the bioaccumulation process. The sampled zebrafish with the wet weight obtained were immediately transferred to a glass beaker with physiological saline for the following procedure to measure the enzyme activities. The control group without exposure to PAHs was also conducted, and the sampling procedure was the same as the bioaccumulation experiment.

1.4. Assays of enzyme activities

The glass beaker with sampled zebrafish was added into icy physiological saline (wet weight of sampled zebrafish: volume of the physiological saline = 1:9). Then the zebrafish were cut off into several organism pieces by medical scissors. The mixture was transferred into a clean centrifuge tubes, and homogenized with ultrasonic tissue destructor (Sonics Uibra Cell VCX 105, Sonics & Materials Inc., USA) in an ice box to obtain the homogenate, followed by centrifuging at 2000 r/min for 10 min at 4°C. The supernatant was transferred into a new centrifuge tube storing at −20°C for enzymatic assay.

The protein concentrations, CYPs, and T-SOD activities were measured by the reagent kit according to the manufacturer's instructions. The concentrations of protein were determined using colorimetric methods with a spectrophotometer (UV-2550, Shimadzu, China) at 595 nm. The activity of CYPs was determined using Elisa Assay with a microplate reader (infinite M200, Tecan (Shanghai) Trading Inc., China) at 450 nm. The activity of T-SOD was determined using colorimetric methods with a spectrophotometer at 550 nm. The results of this enzymatic assay were given in units of CYPs activity per milligram of protein (nmoL/mg protein) and T-SOD activity per milligram of protein (U/mg protein).

1.5. Extraction and analysis of PAHs and zebrafish lipid

The concentrations of PAHs in water samples were measured by solid phase extraction (SPE); the procedure was described in our previous study (Lai et al., 2015). For the determination of PAHs in zebrafish, the detailed information on the extraction of PAHs in zebrafish can be found in our previous study (Xia et al., 2015). Briefly, sampled zebrafish were freeze-dried for 72 hr, and then they were ground in a ceramic mortar to get the homogenate. A total of 0.02 g homogenate was transferred into a 10 mL glass tube added with 8 mL extraction agent (*n*-hexane:dichloromethane = 1:1, *V*/*V*). These tubes were sealed and stored at −4°C for 24 hr, and then bathed in ultrasonic machine (KQ5200DE, Xiren (Shanghai) Material Inc., China) for 30 min. The extract was transferred and another 8 mL extraction agent was added again for the second ultrasonic bath. The extracts of two replications were combined and transferred into 20 mL glass tubes. Subsequently, they were concentrated to less than 2 mL under a gentle nitrogen blow; then the concentrated extracts were filtered with 0.45 μm Teflon membranes; filtrates were concentrated with gentle nitrogen blow to less than 0.5 mL; they were transferred into 2-mL sample vials provided by Agilent. Each vial was added into 50 μL of *m*-terphenyl (1 mg/L) as internal standard, and then diluted with *n*-hexane to 1 mL. These vials were sealed and kept at −4°C before PAH analysis. The concentrations of PAHs were analyzed using a Varian 3800 gas chromatography-4000 ion trap mass spectrometry system equipped with a VF-5 ms column; the detail information was described in our previous study (Shen et al., 2012).

Lipids contents of zebrafish were determined by solvent extraction procedures (Jonker and van der Heijden, 2007). In details, the homogenate of freeze-dried zebrafish was added with 8 mL extraction solvent (*n*-hexane:acetone = 3:1, *V*:*V*) and kept at −4°C for 24 hr; then they were bathed in ultrasonic machine for 30 min. The extract was transferred and another 8 mL extraction solvent was added again for the second ultrasonic bath. The extract of two replications was combined and transferred to a pre-weighed glass tube, in which they were evaporated to dryness under a gentle nitrogen flow. The dry lipid was weighed and the lipid contents of zebrafish were calculated by the mass difference.

1.6. Statistical analysis

All statistical analyses were conducted with OriginPro 8.5 (OriginLab Corp. Massachusetts, USA). The bioaccumulation data were fit into two compartment dynamic model to obtain the kinetic parameters. One-way analysis of variance (ANOVA) was carried out to test the differences between each pair of compared groups, and the difference was considered significant when significance level was less than 0.05. The Pearson correlation coefficient was calculated and used to test the significance of the correlation between each pair of variables.

1.7. Quality assurance and control

The concentrations of PAHs in the blank sample of zebrafish were below the detection limits. The recoveries of phenanthrene and anthracene were 78.7% ± 7.92% and 82.3% ± 6.97% in zebrafish, and 92.3% ± 5.3% and 88.5% ± 6.92% in AFW, respectively (n = 5). The freely dissolved concentrations of PAHs in AFW were measured at the beginning and the end of the experiment, the variations were less than 3% (the results were not shown).

2. Results

2.1. Bioaccumulation kinetics and equilibrium of PAHs in zebrafish

According to the results shown in Fig. 2, the variation of PAH concentrations in zebrafish was similar for phenanthrene and anthracene under each PAH concentration at 10 μg/L in AFW maintained by passive dosing system. The concentrations of PAHs in zebrafish increased rapidly within the initial exposure for 1.5 days and reached the peak value, and then started to decrease until the equilibrium was achieved. The two compartment dynamic model (Landrum, 1989) was applied to describe the bioaccumulation kinetics of PAHs in zebrafish:

$$\frac{dC_b}{dt} = C_w k_u - C_b k_e \quad (1)$$

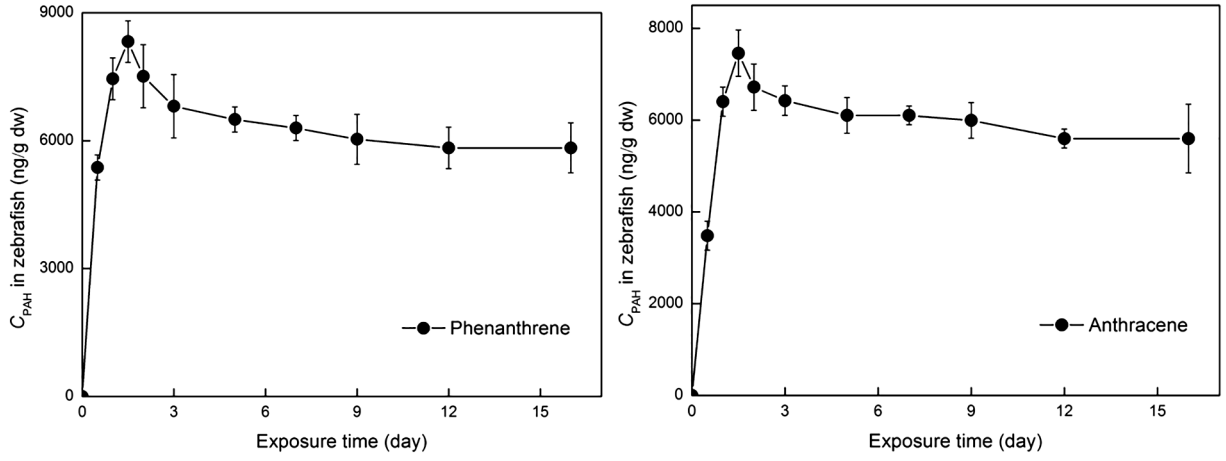

Fig. 2 – The variation of PAH concentrations in zebrafish (mean ± standard deviation, n = 3). dw: dry weight.

Table 1 – Bioaccumulation kinetics parameters in zebrafish and logk_{ow} of two PAHs (mean ± standard deviation, n = 3).

PAHs	k_{u1} (L/(kg·day))	k_{e1} (day^{-1})	k_{u2} (L/(kg·day))	k_{e2} (day^{-1})	BAF (L/kg)	logk_{ow}
Phenanthrene	1647 ± 13	1.86 ± 0.02	286 ± 42	0.49 ± 0.07	583 ± 87	4.57
Anthracene	922 ± 108	0.89 ± 0.23	224 ± 30	0.40 ± 0.09	560 ± 75	4.54

k_{u1} and k_{e1}: the uptake rate constant and the elimination rate constant of the zebrafish in the initial exposure for 1.5 days, respectively.
k_{u2} and k_{e2}: the uptake rate constant and the elimination rate constant of the zebrafish after exposure for 1.5 days, respectively.

where, C_b (μg/kg) is the PAH concentration in zebrafish; C_w (μg/L) is the PAH concentration in AFW; k_u (L/(kg·day)) is the PAH uptake rate constant in zebrafish; k_e (day^{-1}) is the PAH elimination rate constant in zebrafish; and t (day) is the exposure time. The following equation was obtained after integrating the two compartment dynamic model:

$$|C_w k_u - C_b k_e| = e^{-k_e(t-A)} \quad (2)$$

where, A is integral constant. In terms of Fig. 2, uptake process dominated at start of exposure (t < 1.5), and elimination process dominated after the peak value was achieved. Therefore, in the initial 1.5 days, $C_w k_{u1} - C_b k_{e1} > 0$, C_b was obtained as follows:

$$C_b = C_w \frac{k_{u1}}{k_{e1}} \cdot \left(1 - e^{-k_{e1} \cdot t}\right), \quad t < 1.5 \quad (3)$$

After exposure for 1.5 days, $C_w k_{u1} - C_b k_{e1} < 0$, thus C_b was obtained as follows:

$$C_b = C_w \frac{k_{u2}}{k_{e2}} + \left(C_{peak} - C_w \frac{k_{u2}}{k_{e2}}\right) \cdot e^{-k_{e2} \cdot (t-1.5)}, \quad t \geq 1.5 \quad (4)$$

where, k_{u1} and k_{e1} are the uptake and elimination rate constants in the initial 1.5 days, respectively; k_{u2} and k_{e2} are the uptake and elimination rate constants after exposure for 1.5 days, respectively; and C_{peak} is the peak value of PAH concentrations in zebrafish. After fitting into this model, k_{u1} and k_{e1} ($r^2 > 0.992$) as well as k_{u2} and k_{e2} ($r^2 > 0.856$) were obtained (Table 1). In general, k_u was much higher than k_e; k_{u1} and k_{e1} were higher than k_{u2} and k_{e2}, respectively for both phenanthrene and anthracene (p < 0.01).

According to the results shown in Fig. 2, the bioaccumulation equilibrium of PAHs in zebrafish could be reached after exposure for 12 days. The bioaccumulation factor (BAF, L/kg), defined as C_b/C_w (where C_b is the PAH concentrations in zebrafish under equilibrium state, μg/kg dry weight, and C_w is the freely dissolved concentration of PAHs in AFW, μg/L) of phenanthrene and anthracene in zebrafish were obtained, respectively (Table 1). It was consistent with other researches that BAF increased with higher logk_{ow} (Arnot and Gobas, 2006; Mackay et al., 2013; Weisbrod et al., 2007).

2.2. Enzyme activities in zebrafish

Activities of CYPs and T-SOD have been measured in zebrafish, and the results are presented in Fig. 3. The activities of CYPs and T-SOD in zebrafish of the control group without exposure to PAHs are almost constant during the experiment, and CYPs and T-SOD activities in zebrafish with exposure to PAHs are both significantly different from the control group (p < 0.05). The activity of CYPs in zebrafish initially increased rapidly at early exposure period and reached the greatest state within 12 hr, then decreased sharply in the next of 36 hr until the lowest state was achieved, and then increased steadily with time. Finally, the activity of CYPs reached the steady state after exposure for 12 days. The activity of the greatest state was (4.50 ± 0.93) nmol/mg protein, which was a factor of 9 compared with the initial activity before the exposure (0.51 ± 0.11, nmol/mg protein). The activity of the lowest state was (0.51 ± 0.11) nmol/mg protein, which was identical

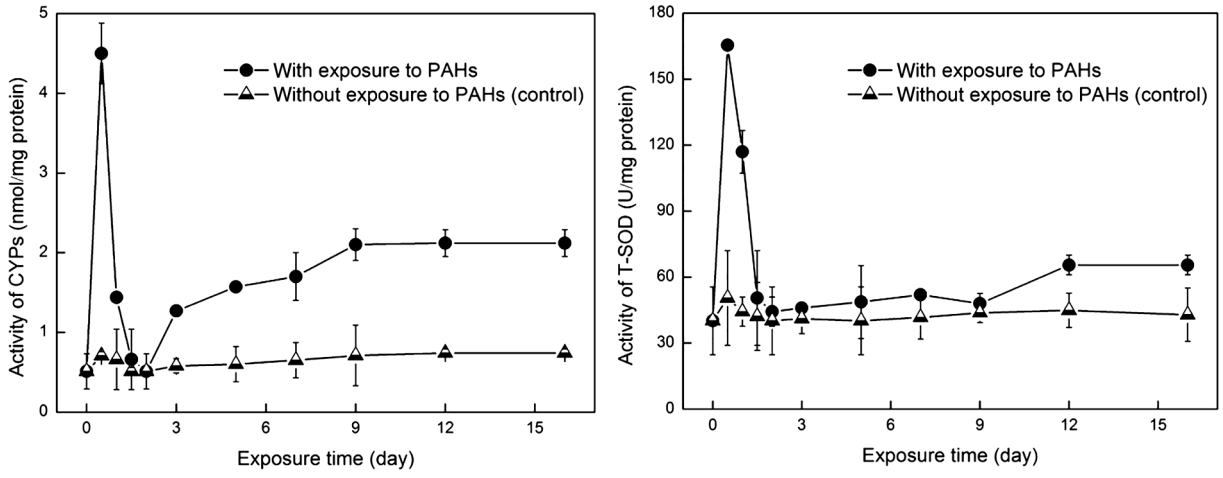

Fig. 3 – The variation of metabolic enzyme activities in zebrafish (mean ± standard deviation, n = 3).

with the initial activity. The activity of the steady state (2.12 ± 0.17, nmol/mg protein) was a factor of 4 compared with the initial activity. This result indicated that the activity of CYPs fluctuated with time during the exposure, but CYPs were activated after exposure to PAHs in general.

The variation of T-SOD activity was similar to that of CYPs activity in zebrafish (Fig. 3). The activity of T-SOD also increased in the first and reached the highest state after exposure for 12 hr, and then decreased with time. The lowest activity was achieved after exposure for 2 days, followed by the increase of the T-SOD activity. The steady state was also reached after exposure for 12 days. The activity of T-SOD at the steady state (65.48 ± 4.51, U/mg protein) was higher than the initial activity (40.12 ± 15.4, U/mg protein), indicating that T-SOD were activated after exposure to PAHs. The activity of T-SOD at the greatest state was (165.98 ± 0.68) U/mg protein, which was a factor of 4 compared with the initial activity. The activity at the lowest state was (44.3 ± 6.65) U/mg protein, which was very similar to the initial activity, but a little lower than the activity at the steady state.

3. Discussion

Several studies have been carried out to investigate the bioaccumulation kinetics of PAHs in aquatic organisms, such as aquatic worm or *Daphnia magna* (ter Laak et al., 2009; Xia et al., 2013), and found that the concentrations of PAHs in these invertebrates increased from the initial exposure until the bioaccumulation equilibrium was reached. This was different from the bioaccumulation kinetics of PAHs in zebrafish observed in the present study (Fig. 2). Actually, the bioaccumulation process observed in this study was also reported by several other researchers (Baussant et al., 2001; Djomo et al., 1996; Sun et al., 2006). For example, Baussant et al. (2001) found that the concentrations of naphthalene, phenanthrene, and chrysene in juvenile turbots, experienced a rapid increase after the start of exposure and reached the maximum level after 3 days, and then a decrease occurred. Sun et al. (2006) studied the bioaccumulation of phenanthrene in *Carassius auratus*, and found that the concentrations of phenanthrene in fish increased rapidly shortly after the start of exposure and reached a maximum level after 2 days, and then it decreased until the steady state was reached. The discrepancy in bioaccumulation kinetics between these studies might be due to the differences in uptake and elimination routes of PAH among aquatic organisms.

In the present study, bioaccumulation of PAHs in zebrafish occurred due to the reason that k_u was much higher than k_e. It is considered that bioaccumulation is caused by the slow elimination process (Jonker, 2012). However, the decrease of PAH concentrations in zebrafish after the peak value may be mainly attributed to the fact that k_{u2} was much higher than k_{u1}.

In order to have a better understanding of bioaccumulation kinetics, it is very important to know the routes contributing to the bioaccumulation of PAHs in zebrafish. Bioaccumulation is a dynamic process including uptake and elimination of PAHs. The uptake process includes dietary and waterborne uptake of PAHs. The dietary uptake of PAHs is due to the consumption of PAHs adsorbed on or accumulated in food, including planktonic organisms, subordinate trophic level organisms, and sediments. Considering that zebrafish was fed for nothing in the present study, dietary uptake of PAHs was not a main route contributing to the bioaccumulation of PAHs in zebrafish. When it comes to waterborne PAHs, only freely dissolved PAHs is taken into consideration for this fraction of PAHs is widely regarded as being bioavailable to the aquatic organisms (Zhang et al., 2014). The bioaccumulation of waterborne PAHs is generally through two primary processes including respiration and osmotic homeostasis regulation, both of which occur by passive diffusion. Since zebrafish is a kind of freshwater fish of which osmotic pressure is higher than that of the surrounding water, it has to maintain the osmotic homeostasis by the way that water will diffuse into zebrafish. Therefore, osmotic homeostasis regulation facilitates PAHs transporting into zebrafish. The main site for respiration and osmotic homeostasis is the gill of zebrafish. It has been reported that the thickness of epithelial cells of the fish gill increased when the fish was exposed to PAHs (phenanthrene and benzo[b]fluoranthene), which are considered as protective measures against toxicants (Martins et al., 2016). Many studies have applied diffusive mass transfer model to predict k_u which is affected by complex function of both biological attributes of the fish and physical properties of the chemical such as k_{ow} of PAHs, the surface area of gills for

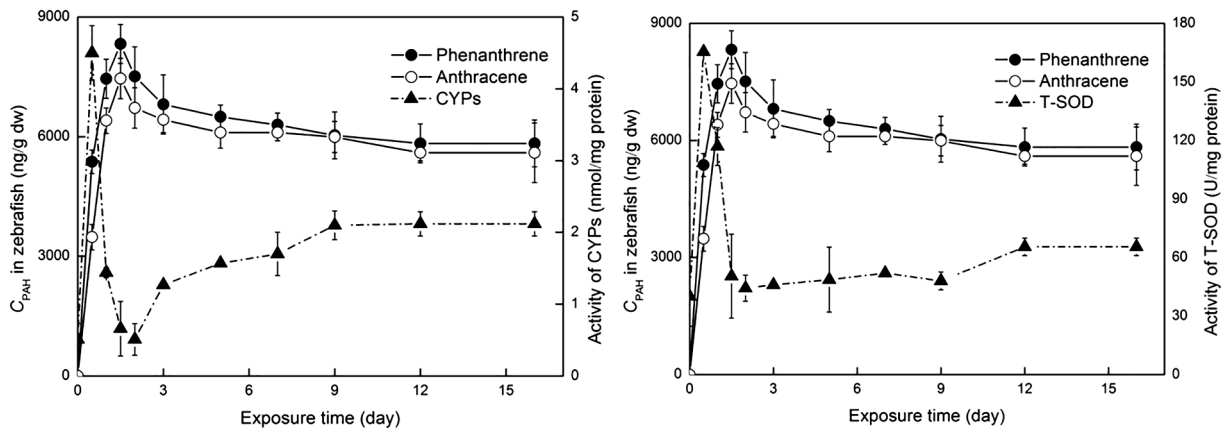

Fig. 4 – The relationship between PAH concentrations and CYPs activities, and T-SOD activities in zebrafish, respectively. CYPs: total cytochrome P450; T-SOD: total superoxide dismutase.

chemical exchange, diffusion length of water, and lipid *etc.* (Kwon and Escher, 2008; Sijm and Vanderlinde, 1995; Xia et al., 2015). The increase in epithelial cell thickness of the gill may increase the diffusion length of PAHs, and further may decrease the k_u. Most studies of bioconcentration were based on the assumption that chemical uptake across the gill dominates all other routes of uptake (Barber, 2003). However, it should be paid attention that water will also diffuse into zebrafish to keep the osmotic homeostasis. And it has also been reported that aquatic organisms can take up contaminants through skin (Glover et al., 2015; Sijm and Vanderlinde, 1995). Notwithstanding, Glover et al. (2015) found that the vast majority of nickel transported across skin was trapped in the skin (93%–98%). The exchange of PAHs through skin cannot be ignored, but the contribution of this process to the total bioaccumulation of PAHs remains unknown. In the present study, the rapid bioaccumulation of PAHs before the peak value may make most of PAHs trapped into the skin, which further hindered the following transportation. This hindrance may also contribute to the decrease of k_u after the peak value.

Elimination process includes metabolism, depuration considered as the inverse uptake process through the gills and skins, excretion, and growth dilution. It has been reported that PAHs can be metabolized by fish through CYPs (Fu et al., 2013; Liu et al., 2014; Oliveira et al., 2007). CYPs, a complex multiple enzyme family, are frequently known as the biotransformation system for organic xenobiotics. Once PAHs enter into aquatic organisms, they will be metabolized by CYPs and bio-transformed into hydrophilic metabolites through different reactions of hydroxylation and conjugation (Torreiro-Melo et al., 2015). The metabolic process will produce reactive oxygen species (ROS) as byproducts, which will induce the antioxidant defense enzymes in cells to protect themselves. Thus, the activities of CYPs and T-SOD can both reflect the extent of metabolism of PAHs in zebrafish.

As shown in Fig. 4, the activity of CYPs reached the greatest level after exposure for 12 hr and then decreased to the lowest level after exposure for 2 days, nearly at the same time, the concentrations of PAHs in zebrafish reached the peak value after exposure for 1.5 days. This result suggested that CYPs were induced once PAHs were accumulated in zebrafish at the start exposure, but the rapid increase of PAHs concentrations can suppress the activity of CYPs. Enzyme activities may be inhibited in the presence of high concentrations of PAHs (Huang et al., 2012; Zhang et al., 2014). The lesions in gills due to the exposure to PAHs may also pose substantial hindering in metabolic routes of fish (Martins et al., 2016). After exposure for two days, the concentrations of PAHs decreased from the peak value until the bioaccumulation equilibrium was reached. At the same time, the activity of CYPs increased from the lowest state until the steady state was achieved. The activity of CYPs at the steady state was higher than the initial state, which indicated that CYPs were activated after exposure to PAHs. The protein contents of zebrafish were increased from initial state to the steady state (Table 2), which may also suggest that CYPs were induced. The increase of the activity of CYPs after the lowest state may be due to self-adaption of zebrafish to the high concentrations of PAHs. It should be paid attention that this increase may lead to the rise of k_{e2} after the peak value compared to k_{e1}. However, k_{e2} was lower than k_{e1} in the present

Table 2 – The wet weight, protein contents, and lipid contents of zebrafish in the exposure period (mean ± standard deviation, n = 3).

Sampling time (day)	0	0.5	1	1.5	2	3	5	7	9	12	16
Wet weight (g)	0.22	0.21	0.21	0.21	0.20	0.21	0.18	0.19	0.16	0.14	0.16
Protein contents (%)[a]	2.78 ± 1.06	3.42 ± 0.51	3.34 ± 0.17	8.66 ± 3.26	7.29 ± 1.08	5.06 ± 1.29	7.3 ± 0.7	6.26 ± 0.95	7.43 ± 0.34	7.84 ± 1.03	7.07 ± 1.04
Lipid contents (%)[a,b]	7.63 ± 0.57				4.07 ± 0.7						3.61 ± 0.7

[a] The protein and lipid contents of zebrafish were carried out in wet weight.
[b] The lipid contents of zebrafish were just carried out in exposure days 0, 2, and 16.

study. This difference was because of the faster decrease of k_u compared with k_e. According to the results shown in Table 1, k_{u1} was nearly six times higher than k_{u2}, whereas k_{e1} was just nearly four times higher than k_{e2} for phenanthrene; k_{u1} was nearly four times higher than k_{u2}, whereas k_{e1} was just two times higher than k_{e2} for anthracene. Thus the rise of k_{e2} caused by the increase of activities of CYPs after the peak value may be counterbalanced by the faster decrease of k_u compared with k_e. And the decrease of k_e may to large extent result from the downfall of k_u according to the diffusive mass transfer model. In addition, since bioaccumulation is considered as a partitioning process between water and lipid (Xia et al., 2015), the decrease of lipid contents of zebrafish shown in Table 2 may also contribute to the drop of the k_u and k_e. The variation pattern of T-SOD activity was similar to that of the CYPs activity. Sun et al. (2006) also found the similar time-dependent response of the T-SOD activity. These findings further suggested that (1) PAHs can be metabolized by zebrafish; and (2) the variation of metabolic enzyme activities had an interactive relationship with PAH concentrations.

Since PAHs can be metabolized by zebrafish, their metabolites will also be eliminated during the elimination process. Parent PAHs can be eliminated through depuration, metabolism, and excretion. The metabolites can also be eliminated through depuration and excretion. The metabolism of PAHs is very complicated which includes phase 1 and phase 2 (Dong et al., 2009). The metabolic process will produce many complex metabolites of which toxicity is different. It has been reported that some metabolites of PAHs in phase 1 are much more toxic than parent PAHs, which are considered as the ultimate carcinogens (Lazartigues et al., 2011; Liu et al., 2014). However, some researches also reported that hydroxy metabolites of PAHs were less toxic than parent PAHs (Sepic et al., 2003). Therefore, further studies should be carried out to investigate the toxicity of PAH metabolites and the contribution of metabolism to the elimination process of PAHs, as well as the dynamic relationship between metabolites and metabolic enzyme activities.

4. Conclusions

In the present study, zebrafish was exposed to the mixture of phenanthrene and anthracene for 16 days under the constant concentration of each PAH maintained by passive dosing systems. The concentrations of PAHs in zebrafish experienced a peak value in the initial exposure, and then decreased until the bioaccumulation equilibrium was reached after exposure for about 12 days. Both of the uptake rate constants (k_u) and the elimination rate constants (k_e) decreased after the peak value. The activities of metabolic enzymes varied with the concentrations of PAHs in zebrafish. In general, the activities of CYPs and T-SOD were induced after exposure to PAHs. The activities of CYPs and T-SOD increased rapidly with the increase of PAH concentrations in the initial stage, but decreased to the lowest state when the concentrations of PAHs reached the peak value. When the bioaccumulation equilibrium of PAHs was achieved, the activities of CYPs and T-SOD also reached the steady state. The decrease of PAH concentrations in zebrafish after the peak value may be due to the great drop of k_u and the variation of CYPs activities. This study suggests that the bioaccumulation kinetics of PAHs and metabolic enzyme activities in aquatic organisms had an interactive relationship, and this should be considered when studying the bioaccumulation equilibrium and kinetics of hydrophobic organic compounds.

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