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Assessment of Bile Fluorescence Patterns in a Tropical Fish, Nile Tilapia (*Oreochromis niloticus*) Exposed to Naphthalene, Phenanthrene, Pyrene and Chrysene Using Fixed Wavelength Fluorescence and Synchronous Fluorescence Spectrometry

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Abstract Bile fluorescence patterns in Nile tilapia, a potential fish for biomonitoring tropical water pollution were assessed following exposure to selected polycyclic aromatic hydrocarbons (PAHs): naphthalene, phenanthrene, pyrene and chrysene. Non-normalized fixed wavelength fluorescence signals in the fish exposed to these PAHs reflected dose and/or time response relationships of their metabolism. Normalizing signals to biliverdin introduced deviations to these response patterns. The optimal wavelength pairs (excitation/emission) for synchronous fluorescence scanning measurements of bile metabolites of naphthalene, phenanthrene, pyrene and chrysene were identified as 284/326, 252/357, 340/382 and 273/382 respectively. This study supports the use of bile fluorescence in Nile tilapia by fixed wavelength fluorescence and synchronous fluorescence spectrometry with non-normalized data as a simple method for screening bioavailability of these PAHs.

Keywords Tilapia · PAH · Biomarker · Bile fluorescence

PAHs are a group of ubiquitous organic pollutants in aquatic ecosystems. PAHs are mainly derived from petrogenic and pyrogenic sources. PAHs have received increased attention recently in pollution studies as some PAHs are highly carcinogenic and mutagenic (Srogi 2007). Sixteen PAHs have been categorized under the group "priority pollutants" which include naphthalene, phenanthrene, pyrene and chrysene. Thus exposure assessments of PAHs in the aquatic ecosystems are important especially in the developing world as the presence of these pollutants poses a serious threat to aquatic resources including fish. In fish, PAHs are biotransformed by mainly liver through oxidation and conjugation reactions to hydrophilic metabolites that are excreted through bile. During the biotransformation process highly reactive intermediate products may be produced which can bind with DNA inducing cancers and mutations. Measurement of biliary fluorescent metabolites is a valid fish biomarker for environmental risk assessment process concerning PAH-contaminant sites (van der Oost et al. 2003). PAH metabolite measurements in bile can be carried out using analytical techniques such as high pressure liquid chromatography, gas chromatography-mass spectrometry (Leonard and Hellou 2001), fixed excitation/emission wavelength fluorescence and synchronous fluorescence spectrometry (Ariese et al. 1993; Aas et al. 2000). Simplicity of the fixed wavelength fluorescence and synchronous fluorescence spectrometry methods makes them suitable for screening recent exposure to PAH on large number of fish rapidly at relatively low cost per sample. Little information is available concerning responses of tropical fish to PAHs. Nile tilapia (*Oreochromis niloticus*) which is a widespread food fish species in tropical countries has been suggested as a test species for biomonitoring of aquatic pollution in tropical environments (Pathiratne et al. 2009). The objective of the present study was to assess the bile fluorescence patterns in Nile tilapia exposed in the laboratory to four commonly occurring PAHs namely naphthalene, phenanthrene, pyrene and chrysene using fixed wavelength fluorescence and synchronous fluorescence spectrometry techniques and to

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evaluate their suitability for assessments of exposure of Nile tilapia to these PAHs.

Materials and Methods

Nile tilapias were obtained from a fish breeding station, National Aquaculture Development Authority, Sri Lanka. Fish were allowed to acclimate to laboratory conditions in fiberglass tanks filled with continuously aerated aged tap water under natural photoperiod for several weeks prior to the experiments. The fish were daily fed with commercial fish food pellets (Prima, Sri Lanka) at 1% of the body weight. During the acclimation period, temperature, pH and dissolved oxygen concentration in water in the tanks ranged from 28 to 30°C, 6.8–7.4 and 3.9–5.2 mg/L respectively. Groups of acclimated tilapias [85–105 g body weight (bw)] were treated with single intraperitoneal injection of naphthalene (20 mg/kg bw) or phenanthrene or pyrene or chrysene (1, 5, 20 mg/kg bw) dissolved in corn oil (1 mL/kg bw) or corn oil only (control groups). After the exposure, the fish were maintained in aerated aged tap water in glass tanks (five fish per 50 L of continuously aerated aged tap water) under natural photoperiod until they are used for biomarker studies. Fish were not fed after the exposure to avoid bile evacuation. PAH exposure levels were based on the levels used in some temperate fish species (Whyte et al. 2000) and designed to ensure a biological response was provoked in this fish species. PAH exposed groups were tested with separate control groups at different time periods. 1 and 3 days after the PAH exposure, control fish and the PAH exposed fish ($n = 5$–6 fish per group) were anesthetized using benzocaine. Bile was taken to a syringe by puncturing the gall bladder and frozen at −80°C until further processing.

PAH metabolites in fish bile were determined by fixed wavelength fluorescence and synchronous fluorescence spectrometry techniques using computer controlled Varian Cary Eclipse fluorescence spectrophotometer with scanning facilities. Two µL of bile diluted in 4 mL of 48% ethanol were used to decrease self absorption and quenching of the fluorescence signal. Fixed wavelength fluorescence at the excitation/emission wavelength pairs 290/335 and 341/383 nm were determined for naphthalene type- and pyrene type- metabolites respectively as described by Aas et al. (2000). Fixed wavelength fluorescence at the wavelength pair 260/380 nm was used for detection of phenanthrene type-metabolites (Krahn et al. 1993). Chrysene metabolites in fish bile were evaluated by the fluorescent method described by Jonsson et al. (2004) and fixed wavelength fluorescence at 272/374 nm wavelength pair was used for detection of chrysene metabolites. For synchronous fluorescence scanning, fluorescence signal recordings of bile samples were taken in the range of 230–500 nm wavelengths with a difference between excitation and emission wavelengths ($\Delta\lambda$) of 42 nm for detection of naphthalene type- and pyrene type- metabolites (Aas et al. 2000). Based on the excitation and emission spectra of the bile of phenanthrene and chrysene exposed Nile tilapia in this study, the $\Delta\lambda$ values 105 and 109 nm were used for detection of phenanthrene type and chrysene type metabolites respectively by synchronous fluorescence spectra (SFS) as optimal sensitivities were detected at these intervals. Synchronous fluorescence spectrometry measurements were also taken at relevant $\Delta\lambda$ values from naphthalene, pyrene, phenanthrene, chrysene and 1-hydroxy pyrene as reference standards (100–200 µg/L). PAHs (purity ≥98%) were obtained from Sigma-Aldrich (USA) and BDH (UK). The fluorescence values were obtained as arbitrary fluorescence units after deducting the signal level of the solvent. Bile intensity was measured at 660 nm in all samples to estimate the biliverdin content (Larson et al. 1947). For the data analysis, the bile fluorescence values of fish exposed to PAH were used as non-normalized or normalized to biliverdin content and the fluorescence responses were analysed separately by one way analysis of variance of log transformed data (ANOVA, $P < 0.05$). Where differences were significant, multiple comparisons were carried out by Tukey's test as appropriate (Zar 1999).

Results and Discussion

The measurement of bile fluorescence has become a popular biomarker to demonstrate the exposure of fish to PAHs. It has been suggested that bile fluorescence data should be normalized to biliverdin or protein concentration in the bile due to the different feeding status of some fish and due to variable water content of the bile. However conflicting data have been published on how to normalize the bile fluorescence (Aas et al. 2000; van den Hurk 2006). In the present study, biliverdin absorption units for 2 µL of bile in Nile tilapia varied within a wide range. Normalizing the fluorescence signals to biliverdin introduced wide variations to these response patterns (results not shown). Dose response and/or time response relationships obtained based on the data non-normalized to biliverdin became statistically not significant when the data were normalized to biliverdin due to high variation. Hence the results are presented as data non-normalized to biliverdin.

Fixed fluorescence determinations in the bile of Nile tilapia (Fig. 1) showed that the PAHs had been metabolized in the fish body and excreted to the bile as corresponding fluorescence values were significantly higher in the bile of PAH exposed fish compared to those in the control fish. In the bile of naphthalene exposed fish, the

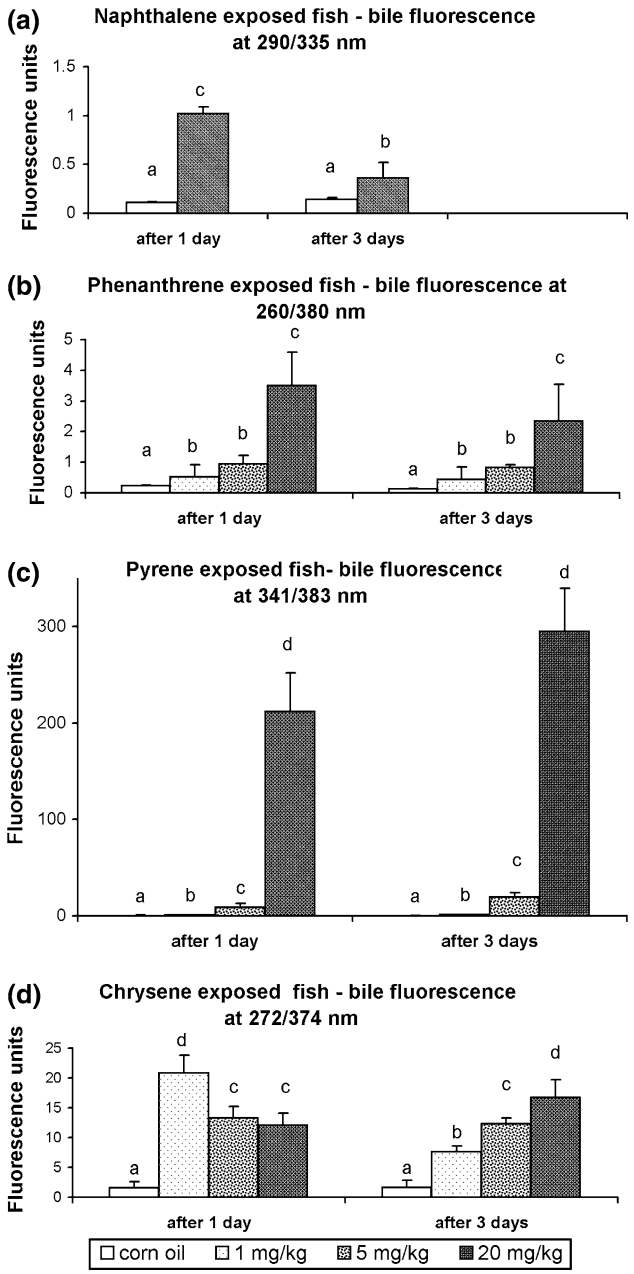

Fig. 1 Fixed fluorescence patterns of bile of Nile tilapia exposed to (**a**) naphthalene (**b**) phenanthrene (**c**) pyrene and (**d**) chrysene. Data are presented as mean and SD of 5–6 fish per group. The *letters* shown in the figure indicates that the means presented with unlike letters are significantly different from each other at $P < 0.05$ for a specific PAH compound (irrespective of the duration of the post exposure)

increase in mean fluorescence values at the wavelength pair 290/335 nm was ninefold compared to those of the controls at 1 day after the exposure where as only threefold increase was observed over the controls at 3 days post exposure indicating rapid metabolism naphthalene in the fish body. In the fish exposed to phenanthrene, corresponding mean fixed wavelength fluorescence values in the bile were increased in comparison to the control fish in a dose dependent manner (after 1 day: 1 mg/kg twofold; 5 mg/kg fourfold; 20 mg/kg 15-fold where as corresponding values after 3 days were 3, 6 and 18-folds respectively). Apparent dose dependent increases in mean fixed wavelength fluorescence signals in the fish exposed to 5 mg/kg phenanthrene were not statistically significant from that of the fish exposed to 1 mg/kg dose due to high variations in the fluorescence signals in the individual fish but both signals were significantly lower than that of the fish exposed to the highest dose of phenanthrene (20 mg/kg bw). Of the four PAHs tested, pyrene showed the strongest fluorescence response. The mean fluorescence units (at 341/383 nm) obtained for the control fish were very low (0.17 and 0.2 at 1 and 3 day respectively). Significant dose–response patterns of mean fluorescence signals compared to control fish were clearly evident in pyrene exposed fish (after 1 day: 1 mg/kg fivefold; 5 mg/kg 50-fold; 20 mg/kg 1,247-fold where as corresponding values after 3 days were 8, 98 and 1,475-folds respectively). In both cases, the increase in fluorescence at 1 day post exposure was not significantly different from the levels at the 3 days post exposure. However, bile fluorescence patterns in chrysene exposed fish were somewhat different from phenanthrene and pyrene. After 1 day, significantly high mean fluorescence signals (13-fold compared to controls) were obtained for the bile of fish exposed to the lowest dose of chrysene (1 mg/kg bw) where as mean fluorescence signals of the bile of the fish exposed to the higher doses of chrysene (5 and 20 mg/kg) were increased only by eightfolds compared to the controls. Nevertheless, a significant dose–response pattern of mean fluorescence signals was observed in chrysene exposed fish after 3 days (1 mg/kg fivefold; 5 mg/kg eightfold; 20 mg/kg tenfold).

SFS of bile from fish exposed to the PAHs (Fig. 2) showed relatively high signals at different regions of the spectra in comparison to control fish. Bile analysis of control fish ensured that non-PAH fluorescence was negligible at the specific wavelengths used for these synchronous wavelength pairs. In the synchronous fluorescence scanning, wavelength maxima and the shape of the peaks obtained for the PAH exposed fish were not clearly distinct from the parent PAHs in the case of naphthalene and phenanthrene but were clearly observable for pyrene and chrysene. Bile of Nile tilapia exposed to naphthalene ($\Delta\lambda = 42$ nm) displayed the maximum peak at 284/326 excitation/emission wavelength pair where as the fluorescence responses of parent naphthalene was optimal at 278/320 nm. In the synchronous fluorescence scan of bile of phenanthrene exposed fish ($\Delta\lambda = 105$ nm), two synchronous peaks were obtained with the highest peak at 252/357 nm where as original phenanthrene exhibited the optimal peak at 242/347 nm. Fixed wavelength

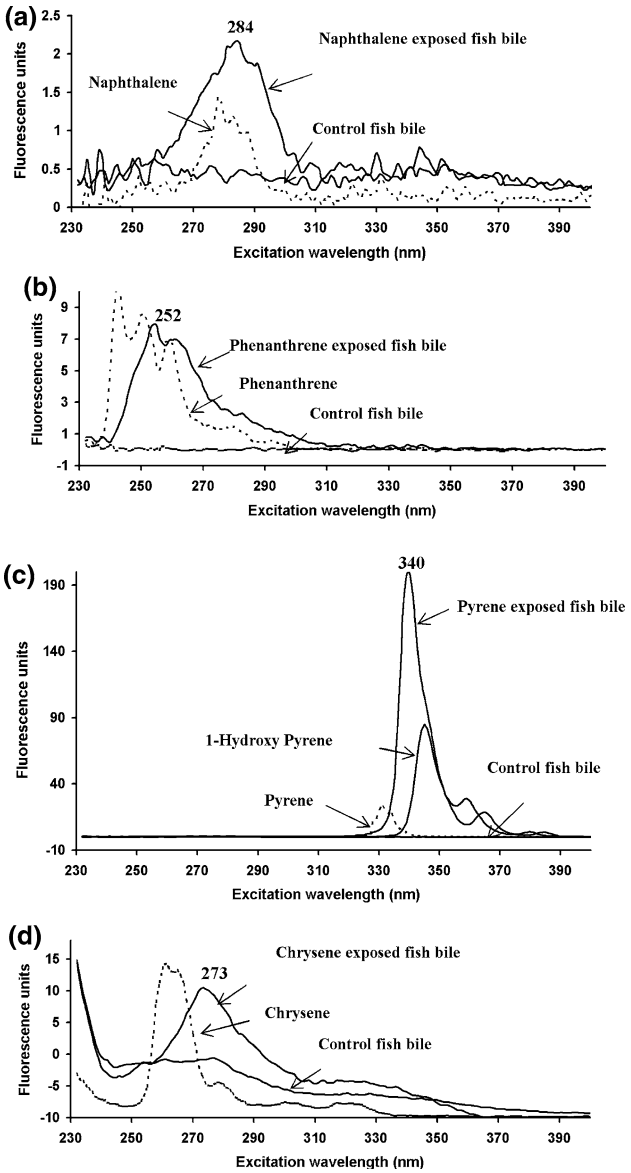

Fig. 2 Synchronous fluorescence spectra (SFS) of bile of Nile tilapia exposed to (20 mg/kg bw) (**a**) naphthalene (**b**) phenanthrene (**c**) pyrene and (**d**) chrysene. SFS scans of control fish bile, 1-hydroxy pyrene and parent naphthalene, phenanthrene, pyrene and chrysene reference standards are also shown for comparison. Excitation/emission wavelength pair maxima for the SFS spectra for parent naphthalene, phenanthrene, pyrene and chrysene reference standards were found at 278/320, 242/347, 331/373 and 261/370 nm respectively

fluorescence at the wavelength pair 260/380 nm used for detection of phenanthrene type- metabolites (Krahn et al. 1993) is somewhat close to the wavelength pairs obtained for Nile tilapia. The optimal peak of synchronous spectra for bile of Nile tilapia exposed to pyrene ($\Delta\lambda = 42$ nm) was at 340/382 nm wavelength pair which is very close to the wavelength pair given by Aas et al. (2000) for pyrene type metabolites. The maximum peak of synchronous spectra for parent pyrene standard was at 331/373 nm wavelength pair. Hence majority of pyrene metabolites excreted into the bile of Nile tilapia fluoresce maximally at a wavelength pair that is different from those of the parent compound. 1-hydroxy pyrene which is one of the products of the phase 1 biotransformation process of pyrene in the fish fluoresced maximally 345/387 nm and showed some overlap with the fluorescence spectrum of the pyrene exposed fish. In the bile of chrysene exposed fish ($\Delta\lambda = 109$ nm), synchronous peak was at 273/382 nm where as original chrysene exhibited the maximum peak at 261/370 nm. The wavelength pair found for chrysene exposed Nile tilapia is close to the optimal excitation/emission wavelength pair (272/374, $\Delta\lambda = 102$ nm) identified by Jonsson et al. (2004) for chrysene type metabolites in some other fish species. The main peaks observed in the Synchronous fluorescence scans of the bile taken from Nile tilapia exposed to naphthalene, phenanthrene, pyrene and chrysene may be mainly due to their glucuronide conjugates as previous studies on some other species indicate that main PAH metabolites in the bile are glucuronides (Varanasi et al. 1981; Leonard and Hellou 2001).

In conclusion, analysis of fixed wavelength fluorescence and synchronous fluorescence spectrometry signals in Nile tilapia showed that the data non-normalized to biliverdin content were able to discriminate bile of fish exposed to selected PAHs namely, naphthalene, phenanthrene, pyrene and chrysene from the bile of respective control fish. The present study supports the use of non-normalized fixed wavelength fluorescence and synchronous fluorescence data in bile of Nile tilapia as a simple and rapid method for screening bioavailability of these PAHs. However, both methods are semi quantitative methods for estimating relative amounts of bile PAH metabolites as other compounds may also fluoresce at these wavelengths. In nature, fish may be exposed to a mixture of different PAH compounds in the wild, and some PAHs may fluoresce in the same wavelength region (Aas et al. 2000). Hence fluorescence responses at specific wavelength pairs obtained for Nile tilapia can only be interpreted semi quantitatively as naphthalene type, phenanthrene type, pyrene type and chrysene type metabolites. The possible interference of PAH signal with biogenic compounds such as cholesterol may equally contribute to the background fluorescence signal for all fish including fish collected from the PAH impacted sites and reference sites.

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