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Synchronous fluorescence spectrometry of 1-hydroxypyrene: a rapid screening method for identification of PAH exposure in tissue from marine polychaetes

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Abstract

The uptake of polycyclic aromatic hydrocarbons (PAHs) by marine deposit-feeding invertebrates can be determined by screening for PAH-derived metabolites. We identified 1-hydroxypyrene as the only intermediate metabolite in tissue of four species of deposit-feeding polychaetes, *Nereis diversicolor, Nereis virens, Arenicola marina,* and *Capitella* sp. I exposed to pyrene spiked sediment. Synchronous fluorescence spectroscopy (SFS) provides a fast and simple method for both qualitative and quantitative analysis of 1-hydroxypyrene in all four species. The SFS assay was validated using HPLC with ultraviolet detection. A good correlation between 1-hydroxypyrene concentrations determined by the two methods was observed. We used HPLC with fluorescence detection combined with enzymatic hydrolysis of conjugated metabolites to investigate species specific metabolite patterns. A tentative aqueous metabolite identification scheme indicates that Nereid polychaetes predominately make use of glucuronide conjugation whereas *Capitella* sp. I. and *Arenicola marina* appear to utilize predominantly sulfate and/or glucoside conjugation. The usefulness of 1-hydroxypyrene as a biomarker for PAH exposure in deposit-feeding invertebrates is discussed.

Keywords: PAH; Marine invertebrates; Bioaccumulation; Metabolism; 1-Hydroxypyrene; *Nereis diversicolor*

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the marine environment (Neff, 1985). PAHs are mainly formed as products from the combustion of fossil fuels but are also natural components of unaltered fossil fuels. The most important inputs of PAH to the marine environment are atmospheric fallout, spillage and seepage of petroleum and oil products, and industrial and domestic sewage. Concern over the fate and effect of PAH is due to their persistence, ability to bioaccumulate, and both acute and long-term toxicity to marine organisms (Cerniglia, 1991; DeWitt et al., 1992; Landrum, Eadie, & Faust, 1991; Swartz, Schults, DeWitt, Ditsworth, & Lamberson, 1990; Weston, 1990). Because some members of the PAH group found in the environment are also procarcinogens (Menzie, Potocki, & Santodonato, 1992), there is additional concern about trophic transfer of PAH residues and biotransformation products with ultimate exposure to humans.

PAHs are primarily bound to sediment particles and are readily accumulated by marine deposit-feeding invertebrates (Meador, Stein, Reichert, & Varanasi, 1995). Marine polychaetes have known PAH metabolic capabilities (Fries & Lee, 1984; Giessing, Mayer, & Forbes, 2003; McElroy, Leitch, & Fay, 2000; McElroy, 1985), though the ability to metabolize PAH varies widely within and among phyla and rates are much lower than those observed in vertebrates (Livingstone, 1994). Metabolism of PAH in marine invertebrates is apparently related to the cytochrome P-450 enzyme suite which converts hydrophobic, lipid-soluble, parent PAll to water soluble metabolites, but the mechanism by which the involved enzymes are regulated are still poorly understood (Hahn, 1998).

Routine monitoring of PAH in the marine environment usually involves the determination of parent PAHs in sediment samples. Benthic invertebrates are often used to assess the toxicity, bioavailability, and the potential trophic transfer of PAHs from sediments (Chapman & Wang, 2001; Di Toro et al., 1991; Næs, Hylland, Oug, Forlin, & Ericson, 1999). However, chemical analysis of invertebrates provides little useful information for compounds that are rapidly metabolized by some species and information on species-specific PAH metabolic capacity is therefore critical in evaluating bioaccumulation data. Effects of PAHs are mainly caused by their metabolites rather than by parent compounds (James & Kleinow, 1993; Livingstone, 1993). Thus simple methods for detection and quantification of PAH metabolites in marine invertebrates may serve as useful screening tools for preliminary stages of environmental risk assessment of PAH-contaminated sediment.

Although the PAH pyrene is not known for its toxicity, it is one of the most dominant PAHs in the marine environment, and it usually co-occurs with other PAHs that are metabolized into toxic products. Furthermore, 1-hydroxypyrene is the only known intermediate metabolite of pyrene observed in invertebrates such as the marine deposit-feeding polychaete *Nereis diversicolor* (Giessing et al., 2003; Giessing & Lund, 2002) and the terrestrial isopod *Porcellio scaber* (Stroomberg, Knecht, Ariese, Gestel, & Velthorst, 1999) as well as in mammals and fish (Ariese, Kok, Verkaik, Gooijer, Velthorst, & Hofstraat, 1993c; Boyland & Sims, 1964; Jacob et al., 1989; Keimig, Kirby, Morgan, Keiser, & Hubert, 1983). Measurement of l-hydroxypyrene in

excretory products of animals and humans has gained considerable attention as a potential biomarker for PAH exposure and is widely used as a biomarker to study human PAH exposure (Levin, 1995; Schaller, Angerer, & Hausmann, 1993).

Biomarker studies using l-hydroxypyrene have previously dealt with liquid matrices such as urine and fish bile (e.g. Lin, Cormier, & Racine, 1994; Schaller et al., 1993). This approach cannot be utilized when dealing with small tissue samples from marine polychaetes and a more elaborate liquid:liquid partitioning scheme is usually applied (e.g. Christensen, Andersen, & Banta, 2002; McElroy et al., 2000). Even though conventional fluorescence spectroscopy has excellent sensitivity in determining trace PAH, its application to mixtures of fluorescent compounds is limited (Santana Rodriguez & Padrén Sanz, 2000). This limitation is especially evident when the compounds have high structural similarity, as do pyrene and 1-hydroxypyrene. Such spectral interferences can sometimes be resolved by using synchronous fluorescence spectroscopy (SFS). Scanning both the excitation and emission monochromator with a constant wavelength difference, rather than scanning the excitation monochromator while keeping the emission constant, results in an important simplification of the fluorescence spectra and in the ideal case only one spectral element remains (Vo-Dinh, 1982). By reducing spectral interference, individual components of moderately complex mixtures can be identified with limited sample preparation and without chromatographic separation.

We report here a simple fluorometric method that allows rapid detection and quantification of 1-hydroxypyrene in small samples of tissue extracts from *Nereis diversicolor* exposed to sediment-associated pyrene. We have used this method for qualitative analysis of pyrene metabolism in three other species of marine deposit-feeding polychaetes, *Arenicola marina, Capitella* sp. L, and *Nereis virens*. High pressure liquid chromatography with ultraviolet (HPLC/UV) and fluorescence (HPLC/F) detection was used as a complementary, quantitative method for confirmation of the SFS results of *Nereis diversicolor* and to investigate species-specific metabolite conjugation patterns respectively.

2. Materials and methods

Pyrene (98%), 1-hydroxypyrene (98%), and Na_2SO_4 (>99%) were purchased from Aldrich Chemical Company (Steinheim, Germany). HPLC grade acetonitrile, hexane, chloroform and methanol (all Chromosolv® grade) were acquired from Reidel-deHaën AG (Seelze, Germany). HPLC-grade water was obtained from Merck (Darmstadt, Germany). Glucuronidase-arylsulfatase (from *Helix pomatia,* EC 3.2.1.31 and EC 3.1.6.1. 30 and 60 U/ml respectively) was supplied by ICN Biochemicals (Aurora, OH, USA). All chemicals were used as received.

2.1. Test animals

Individual *Nereis diversicolor* were collected on a sand flat in the Damariscotta River, South Bristol, Maine, USA in June of 1999 and July 2000 and at Store

Havelse, Roskilde Fjord, Denmark in October and November of 1999. Gravid females were avoided. Three additional species of marine polychaetes, *Arenicola marina, Capitella* sp. I, and *Nereis virens,* were tested for pyrene metabolism. *Arenicola marina* was collected at Store Havelse, Denmark at the same time as *Nereis diversicolor*. *Capitella* sp. I was from a mature culture (20 year old) obtained from SUNY, Stony Brook (originally identified to sibling species by J. P Grassle, Rutgers University New Jersey, USA). Cultured *Nereis virens* was purchased from a commercial worm supplier in Roskilde, Denmark. All animals were kept in culture in 1-mm sieved sediment and with 0.45 μm filtered seawater for a minimum of 7 days prior to use.

2.1.1. Sediment spiking

The following procedure was followed in all sediment contaminations. Sediment was collected at the same time as worms, sieved (1 mm) and frozen (−18 °C) prior to use. Thawed sediment was contaminated with pyrene by adding the desired amount of pyrene dissolved in a minimum of acetone to a slurry of sediment and 0.45 μm filtered seawater. Seawater used in the experiments was always filtered unless otherwise stated. The slurry was placed on a stand with a power drill, fitted with a mud slinger, and was mixed continuously for a minimum of 24 h. The slurry was then allowed to settle, the water was decanted, and the sediment was allowed to sit at 5 °C for 1 week prior to use in microcosms.

2.1.2. Accumulation of pyrene in Nereis diversicolor

We investigated accumulation of pyrene and induction of pyrene metabolism over time in *Nereis diversicolor* by exposing worms to 136 μg pyrene g^{-1} sediment (dw) for a total of 9 days. Six separate plastic containers, each with five worms and 500 ml pyrene-contaminated sediment, were placed in a tank in a flow-through system with running seawater. One container was sacrificed daily on day 1 through five and one was sacrificed on day 9. Two separate containers containing five worms each were kept in a separate system as non-exposed references to be sampled at the beginning and end of the experiment. Pyrene and 1-hydroxypyrene were identified and quantified using SFS and traditional fluorescence spectroscopy.

Concentration-dependent pyrene metabolism in *Nereis diversicolor* was investigated by exposing worms to sediments containing 1, 20,50 and 100 μg pyrene g^{-1} (dw). Five worms in each treatment were exposed in a 3 l plastic container with 500 ml sediment and 1 l 0.45 μm filtered sea water for 5 days. A separate container with five worms was kept in clean sediment for reference. Each container was fitted with an airstone and bubbled continuously through the experiment. Fresh filtered seawater was added daily. Tissue samples were extracted as described below and analyzed for pyrene and 1-hydroxypyrene using HPLC/UV and SFS.

3. Sample treatment

Accumulation experiment: worms that were collected on days 1–5 and 9 were allowed to defecate in individual petri dishes with seawater for a minimum of 12 h, a

time sufficient to purge their guts of sediment prior to extraction (Kane Driscoll & McElroy, 1996). The worms were weighed and transferred to test tubes and 2 ml 50:50 methanol/water was added. The worms were then homogenized using a Tissue Tearor (Biospec Products, Inc. Bartlesville, OK, USA) at full speed for 1 min and subsequently adding 2 ml chloroform. Elimination of aqueous metabolites through gut fluid is expected as an aqueous intermediary necessary for excretion (Giessing et al., 2003). Though handled delicately, some of the worms squirted gut fluid making the aqueous metabolite pool of whole worm homogenate difficult to handle quantitatively. Thus to avoid interference from aqueous metabolites containing the 1-hydroxypyrene fluorophore, whole worm homogenates were extracted in chloroform prior to quantification of l-hydroxypyrene using SFS. The samples were sonicated for 10 min and centrifuged at 500 *g* for 10 min to precipitate any debris and the chloroform phase was removed. This procedure was repeated twice to a total chloroform volume of 6 ml. The chloroform phase was then dried over anhydrous sodium sulfate, blown down to dryness under a gentle stream of nitrogen gas and redissolved in 1 ml methanol.

Concentration series experiment: Tissue extraction was performed by homogenizing whole worms in 2 ml methanol using a Tissue Tearor at full speed for 1 min and subsequently sonicating samples for 10 min. Samples were then centrifuged at 500 *g* for 10 min to precipitate any debris. Supematant (500 μl) was filtered through a 0.22 μm syringe filter and transferred directly to brown HPLC vials without any further sample preparation. The samples were also analyzed using SFS without any further sample preparation.

Total metabolite pattern experiment: Tissue samples from *Nereis diversicolor, Arenicola marina, Capitella* sp. I, and *Nereis virens* were prepared as described above. Due to the large size of *Arenicola marina* and *Nereis virens* their gut tissue was substituted for whole worm homogenate. Unlike other marine invertebrates such as mollusks and crustaceans, annelid worms do not have an easily separable digestive gland. Annelids have specialized tissue (chloragogen) associated with the alimentary tract, the function of which is comparable to the mussel digestive gland. Furthermore, the enzymes involved in biotransformation of contaminants is located in the endoplasmatic reticulum of chloragog tissue of polychaetes (Lee, 1981). Thus, due to the size and morphology of *Nereis diversicolor* and *Capitella* sp. I, it is not possible to separate the 'reactive' tissue for analysis. Samples were analyzed using HPLC/F.

3.1. Enzymatic hydrolysis

The presence of glucuronic acid and sulphate conjugates of 1 -hydroxypyrene in tissue samples was studied by enzymatic hydrolysis of tissue samples. Glucuronidase-arylsulfatase enzymes effectively hydrolyse glucuronide and sulfate conjugates to give free 1-hydroxypyrene. Tissue methanol extracts were diluted 1:10 with 0.22 μm filtered deionized water (DI) and incubated for 2 h at 37 °C with 10 μl of glucuronidase-arylsulfatase solution. After addition of 500 μl ice-cold 100% ethanol the samples were mixed and centrifuged at 3000 *g* for 5 min to precipitate denatured

protein. The supernatant was transferred to brown HPLC vials and analyzed using HPLC/F.

3.2. Synchronous fluorescence spectroscopy

Accumulation of pyrene in *Nereis diversicolor* was followed using SFS with a constant wavelength difference of 34 mm between excitation and emission wavelengths channels. Samples were diluted 1:10 with methanol prior to analysis. SFS was performed on a Hitachi F-4500 spectrofluorometer using a 1 cm methyl acrylate cuvette (VWR, Bridgeport, NJ, USA), with 5 nm slit widths on the emission and excitation channels, a PMT voltage of 700 V and a response time of 0.1 s. The diluted samples were not deoxygenated and consecutive dilutions of samples gave a proportional decrease in peak height (data not shown). The fluorescence life time of 1-hydroxypyrene is short compared to that of pyrene and does not depend critically on the presence of oxygen (Ariese, Kok, Verkaik, Gooijer, Velthorst, & Hofstraat, 1993a). Calibration curves were made by standard additions of pyrene and 1-hydroxypyrene in methanol to a tissue sample of an unexposed worm. Quantification was done by measuring peak heights and not area because this approach was less influenced by spectral overlap. Calibration curves had regression coefficients $r^2 > 0.98$ for both pyrene and 1 -hydroxypyrene. SFS identification of compounds containing the 1-hydroxypyrene fluorophore in tissue samples *of Nereis virens, Capitella* sp. I. and *Arenicola marina* exposed to pyrene spiked sediment was done as described above.

3.3. High pressure liquid chromatography

Quantification of pyrene and 1-hydroxypyrene in tissue samples from *Nereis diversicolor* in the concentration series experiment was done by HPLC/UV. The method consisted of an acetonitrile/water gradient scheme using a reverse phase Supelcosil LC-PAH column, 15 cm×4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA).The acetonitrile/water *(v/v)* gradient profile used for quantification of pyrene and 1-hydroxypyrene was 50:50 for 5 min, 100:0 over 20 min, and 100:0 for 8 min at a flow rate of 0.85 ml mm^{-1}. Column temperature was kept at 28 °C. The system consisted of two Gilson 306 solvent pumps, a Gilson 831 Temperature Regulator, a Gilson 234 Autoinjector, and an ERC 3415 degasser. The injection volume was 100 μl on a 500 μl stainless steel injection loop. Detection was performed with a Gilson 119 UV/vis detector by measuring absorbance at 339 nm. Quantification was done by measuring peak area using external calibration curves of pyrene and 1-hydroxypyrene standards in acetonitrile. Calibration curves had regression coefficients $r^2 > 0.98$ for both pyrene and 1-hydroxypyrene.

Total metabolite pattern was analyzed on a Hitachi D-7000 HPLC using a method optimized for pyrene metabolites originally provided by Dr. Gerard Stroomberg (AquaSence, Amsterdam, NL). The method is described in detail in Giessing and Lund (2002); in brief a Vydac C18 reverse phase column 25 cm×4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) was used with an acetonitrile/10 mM

ammonium acetate (pH = 5) (v/v) gradient. The gradient profile was 5/95 directly to 90/10 over 40 min, and then hold 90/10 for 10 min at a flow rate of 0.8 ml min^{-1}. Column temperature was kept at 28 °C. Detection was performed by measuring absorbance at 339 nm and fluorescence $\lambda_{EX/EM} = 346/384$ nm for metabolites/conjugates and $\lambda_{EX/EM} = 333/384$ nm for parent compound. Injection volume was 100 μl. Peaks were identified with a Hitachi L-7450 Diode Array Detector (DAD).

4. Results and discussion

Fig. 1 shows classical fluorescence (Em 430 mm) and SFS ($\Delta\lambda = 34$ nm) spectra of pyrene and 1-hydroxypyrene standards, and tissue extracts from a *Nereis diversicolor* exposed for 5 days to 136 μg pyrene g^{-1} sediment (dw). SFS simplifies the fluorescence spectra of a pyrene and 1-hydroxypyrene mixture (Fig. 1a) and pyrene and 1-hydroxypyrene are readily identified at 339 nm and 355 nm respectively (Fig. 1b). The synchronous fluorescence spectra of exposed *Nereis diversicolor* (Fig. 1c) showed two peaks at 350 nm and 338 mm analogous to the spectrum of the 1-hydroxypyrene and pyrene standard in Fig. 1b. Spectra of unexposed worms showed no peaks other than the broad protein band at ~280 nm with varying intensity (Mayer, Schick, & Loder, 1999). The small blue shift observed in the spectrum of tissue sample can be explained by the presence of conjugated 1-hydroxypyrene in the sample. Ariese et al. (1993a) reported that the spectrum of glucuronide-conjugated 1-hydroxypyrene was blue-shifted 5 nm and was 2-fold more intense than that of free 1-hydroxypyrene. Generally, an increase in the extent of aromatic structure leads to a shift of absorption and fluorescence spectra to longer wavelengths and to an increase in fluorescence intensity. Two major metabolites, 1-hydroxypyrene and a pyrene-1-glucuronide conjugate, have been identified in tissue extracts of *Nereis diversicolor* exposed to sediment-associated pyrene (Giessing & Lund, 2002), with pyrene-1-glucuronide constituting >70% of total pyrene-derived compounds measured in tissue (Giessing et al., 2003). The peak labeled 1-hydroxypyrene in Fig. 1c is the signal from all compounds containing the l-hydroxypyrene fluorophore and thus the combined fluorescence signal from 1-hydroxypyrene, pyrene-1-glucuronide and possibly other minor 1-hydroxypyrene conjugates (e.g. sulfates and glucosides).

The worms reached quasi-steady state tissue concentrations of both pyrene and 1-hydroxypyrene after approximately 3 days of exposure to a high spike of pyrene (Fig. 2). Subsequently, an exposure period of 5 days was chosen in later experiments for all species studied. Similar quasi-steady state tissue concentration within 2–3 days of exposure to PAH has previously been reported for deposit feeding marine polychaetes (Forbes, Forbes, & Holmer, 1996; Kure, 1997).

The worms showed increasing accumulation of pyrene with increasing exposure concentration up to 50 μg g^{-1} sediment followed by lower accumulation at 100 μg pyrene g^{-1} sediment (Fig. 3). The concentration of both 1 -hydroxypyrene and pyrene was below detection in samples of unexposed worms (data not shown). There were highly significant positive correlation between the measured pyrene and 1-hydroxy-

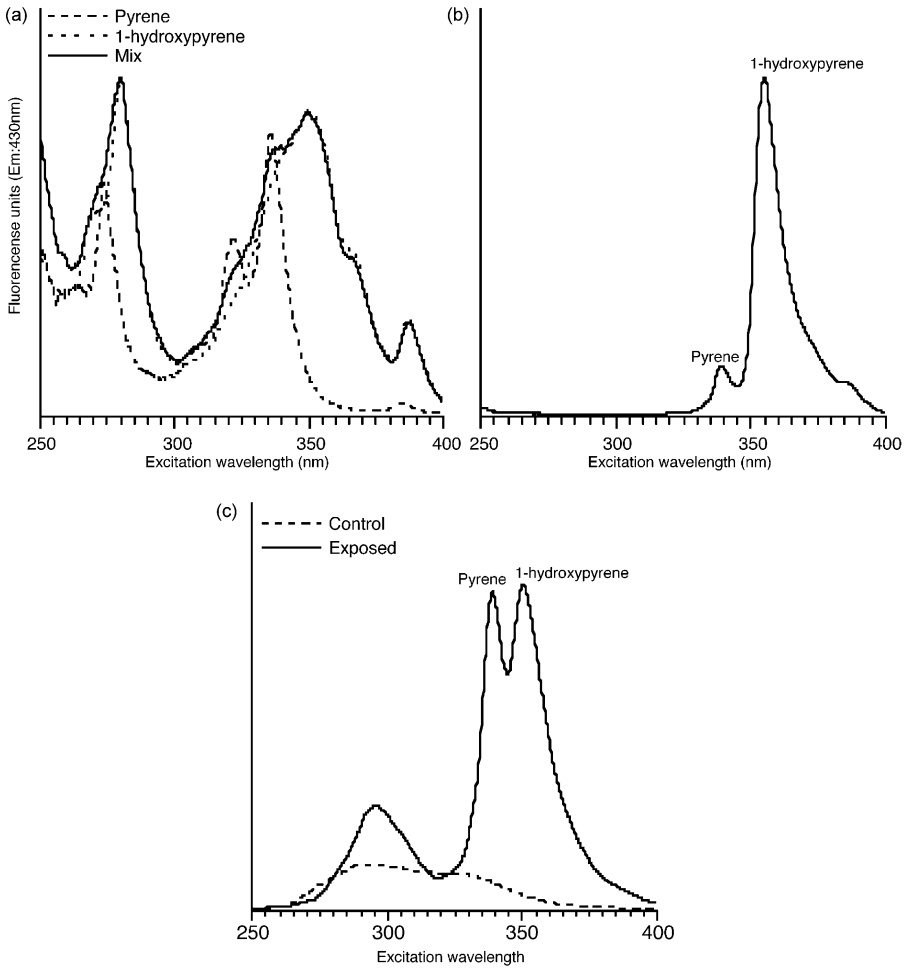

Fig. 1. Fluorescence spectra of pyrene and 1-hydroxypyrene. **a:** Traditional fluorescence spectrum of pyrene (30 ng/ml) and 1-hydroxypyrene (10 ng/ml) standards scanning the excitation channel while measuring emission at 430 nm. **b:** Synchronous fluorescence spectrum (SFS, $\Delta\lambda = 34$ nm) of the same mixture of pyrene and 1-hydroxypyrene. **c:** SFS spectrum ($\Delta\lambda = 34$ nm) of tissue extract from *Nereis diversicolor* exposed to pyrene contaminated sediment.

pyrene concentrations (Spearmans rank-order correlation, $P < 0.01$). As mentioned earlier the samples presented in Fig. 2 were extracted to eliminate the influence of conjugated 1-hydroxypyrene prior to quantification using SFS. Lower pyrene accumulation at a higher exposure concentration is confirmed by adding the data for day 5 from Fig. 2 to the data in Fig. 3 (data points with open legends). The apparent decrease in pyrene accumulation above exposure concentrations of 50 μg pyrene g^{-1} sediment is possibly due to changes in *Nereis diversicolor* feeding behavior, such as a decrease in feeding rate. When exposed to 16 μg g^{-1} fluoranthene the feeding rate of *Arenicola marina* showed an exponential decline with increasing exposure concentration and was

reduced to 4% of that of control worms (Kure, 1997). Data on changes in feeding rate in *Nereis diversicolor* under pyrene exposure are lacking, but the bell shape of the accumulation curve seems to suggest that there is a threshold concentration below which *Nereis diversicolor* metabolic capacity can keep pyrene tissue concentrations below acute toxic levels. However, this conclusion is somewhat speculative and the

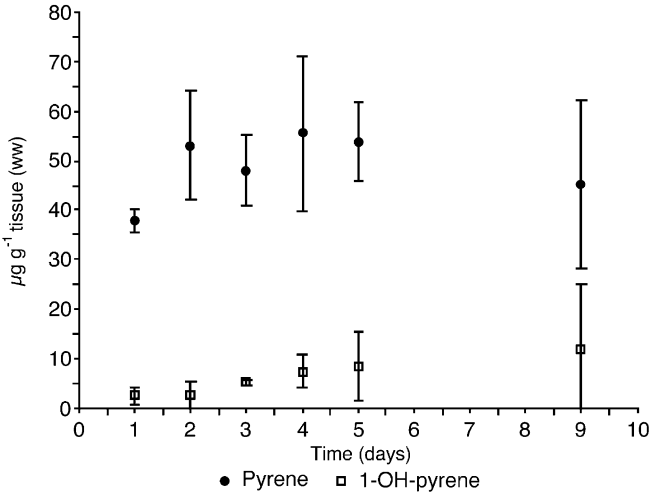

Fig. 2. Accumulation of pyrene and formation of 1-hydroxypyrene in *Nereis diversicolor* exposed to 136 μg pyrene g^{-1} sediment (dw) measured by SFS. Data are presented as average ± standard deviation for visualization purposes, $n = 5$ except for day 1 and day 3, $n = 3$.

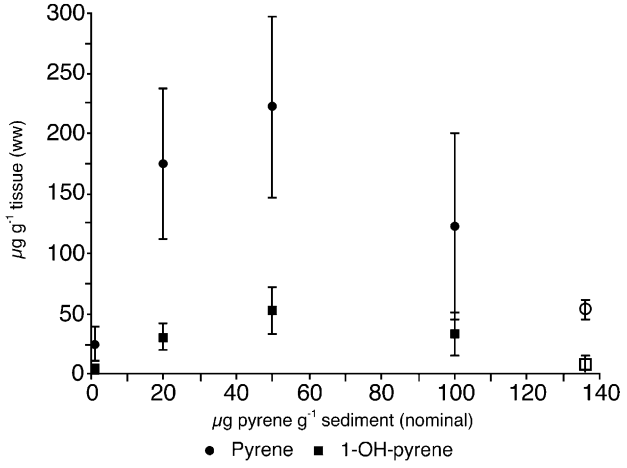

Fig. 3. Concentration of pyrene and 1-hydroxypyrene in *Nereis diversicolor* exposed to 1,20, 50 and 100 μg pyrene g^{-1} sediment (dw) measured by HPLC/UV. Data are presented as average ± standard deviation, $n = 5$ except for exposure concentrations 50 and 100 μg g^{-1} sediment were $n = 4$. The open data points at 136 μg pyrene g^{-1} sediment are the averages ± standard deviations of day 5 from the data presented in Fig. 2.

influence of pyrene on the feeding behavior of *Nereis diversicolor* warrants further investigation.

Tissue concentrations of 1-hydroxypyrene from 20, 50 and 100 μg pyrene g^{-1} sediment treatments in the concentration series experiment (Fig. 3) were also quantified using SFS without extraction prior to analysis. There was a positive correlation (though not significant, Spearmans rank-order correlation, $P = 0.081$) between 1-hydroxypyrene concentrations measured by the two methods with SFS concentrations being on average 1.7 times higher than HPLC—all but one of the data points are above the 1:1 line on Fig. 4. As mentioned earlier the SFS signal measured is the combined signal of all compounds containing the 1-hydroxypyrene fluorophore. Ariese et al. (1993a) reported a 2.2-fold smaller fluorescence yield of free 1-hydroxypyrene compared to 1-hydroxypyrene glucuronide. Thus SFS-determined 1-hydroxypyrene concentrations (i.e. Σ of all 1-hydroxypyrene containing species) would be expected to be higher than HPLC. Lin et al. (1996) observed a similar difference when comparing fixed wavelength fluorescence and HPLC/F, using a linear acetic acid-water and methanol gradient program, for determining benzo[*a*]pyrene (BaP) exposure in fish bile. In another fish bile study, high correlation between BaP-type metabolites measured by HPLC/F and pyrene-type metabolites measured by SFS indicated the suitability of using the latter as a surrogate for exposure to BaP-type PAHs (Lin et al., 1994). Inaccurate results for PAH content of polychaetes from the New York Bight have been reported when analyzed using only spectrofluorometric techniques (Farrington, Wakeham, Livramento, Tripp, & Teal, 1986). The inaccuracy was due to fluorescent octahydrochrysenes, presumably of microbial origin, accumulated by the polychaetes and not related to

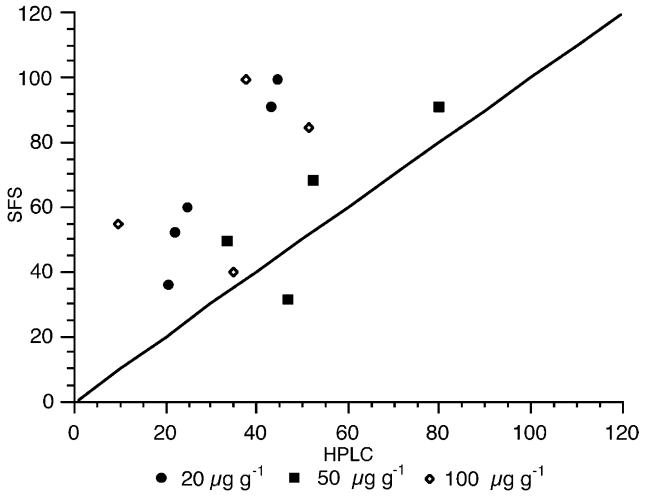

Fig. 4. Correlation between 1-hydroxypyrene concentrations (μg g^{-1} tissue) measured by SFS and HPLC/UV. The straight line indicates the 1:1 ratio and exposure concentration is indicated by the legend on the figure. There was a good correlation though not significant ($P = 0.0814$) between concentrations measured by the two methods.

petroleum contamination of the study site. A similar interference does not confound the present experiments but should be addressed when studying pyrene metabolism in marine invertebrates collected from oil contaminated areas. Thus, future studies using the SFS method for screening of PAH contaminations, should be verified using more sophisticated techniques such as HPLC or GC/MS and should employ enzymatic hydrolysis of the samples prior to quantification.

Fig. 5 shows SFS spectra of methanol extracts of tissue from *Nereis diversicolor, Nereis virens, Capitella* sp. I. and *Arenicola marina* exposed to sediment-associated pyrene. *Nereis diversicolor* and *Capitella* sp. I. showed two distinct spectral elements, pyrene and 1-hydroxypyrene-type metabolites, in the 340–350 nm region. SFS spectra *of Nereis virens* and *Arenicola marina* only showed one peak that can be related to pyrene metabolism i.e. the 1-hydroxypyrene peak at ≈350 nm. Lack of a pyrene peak is due to rapid turnover of pyrene to 1-hydroxypyrene at the lower exposure concentration (5 μg^{-1} as opposed to 136 and 50 μg pyrene g^{-1} sediment)

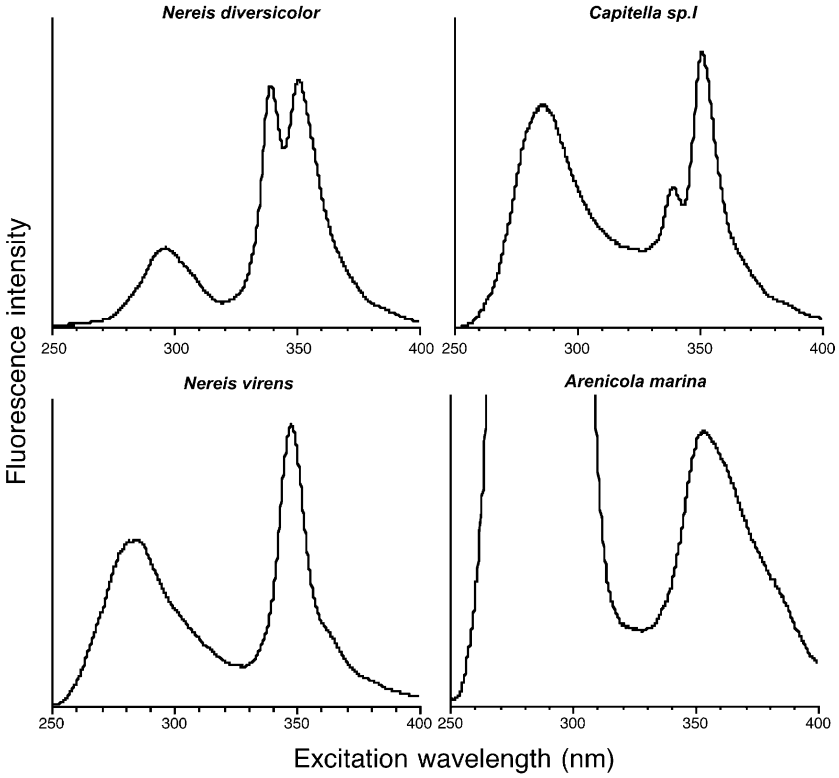

Fig. 5. Synchronous fluorescence spectroscopy ($\Delta\lambda = 34$ nm) of tissue extracts from four species of marine polychaetes (*Nereis diversicolor, Nereis virens, Arenicola marina,* and *Capitella* sp. I.) exposed to sediment-associated pyrene. The peak from 1-hydroxypyrene-type metabolites at ≈350 nm, is readily identified in all four species.

and to quenching by the complex sample matrix. Both worms have intense protein peaks around 280 nm.

Identification of 1-hydroxypyrene as the only metabolic intermediate of pyrene in all four species of worms is corroborated by the HPLC/F chromatograins (Fig. 6). Lower chromatograms marked *a* are diluted samples identical to those presented in Fig. 5 and upper chromatograms marked *b* are the same sample after enzymatic hydrolysis with glucuronidase-arylsulfatase. In all four species, treatment with glucuronidase-arylsulfatase caused a significant increase in 1-hydroxypyrene signal at 32.5 min. The chromatograrn of *Nereis diversicolor* before hydrolysis show three peaks at 19.0, 21.7 and 22.2 min. The peak at 19.0 mm has previously been identified as 1-hydroxypyrene glucuronide (Giessing & Lund, 2002) and it is, together with the small peak at 22.2 min, hydrolysed after treatment with glucuronidase-arylsulfatase. The peak at 22.2 mm is therefore likely a sulfate conjugate. The peak at 21.7 min is not affected by glucuronidase-arylsulfatase treatment. Glucuronidase is highly specific for the carbohydrate part and it hydrolyzes neither β-glucosides nor α-glucusiduronic acids (Tomasic & Keglevic, 1973). Glucoside and sulfate conjugations are thought to predominate over glucuronic acid conjugation in marine invertebrates (Livingstone, 1998). Using this commonly observed trend the nonhydrolysable peak at 21.7 mm is tentatively suggested to be a glucoside conjugate. The tentative identification has, after this paper was submitted, been corroborated by preparations of glucuronide, glucoside, and sulfate conjugated 1-hydroxypyrene using gut tissue from *Nereis virens* (Jørgensen and Giessing, unpublished data) and will be presented in a forthcoming paper. Previous studies of pyrene metabolism in *Nereis diversicolor* have shown that other aqueous metabolites might be present but are most likely insignificant in the mass balance of pyrene-derived compounds (Giessing et al., 2003). However, conjugates of organic pollutants other than carbohydrate and sulfate (e.g. glutathione and amino acid) have been indicated in marine invertebrates (James, 1987) and their presence in tissue of the worms presented here cannot be excluded.

Metabolism of PAH in marine invertebrates is apparently related to the cytochrome P450 enzyme suite, which converts hydrophobic, parent PAH to water soluble metabolites in a two phase process. The enzymes of Phase I metabolism introduce a functional group (OH, COOH, NO_2 etc.) into the PAH, to which Phase II enzymes attach a large polar moiety (e.g. carbohydrates, glutathione, sulfate, amino acids) to facilitate excretion. *Nereis diversicolor, Nereis virens,* and *Capitella* sp. I. all have known cytochrome P450-like PAH metabolic capabilities (Forbes, Andreassen, & Christensen, 2001; Giessing & Lund, 2002; McElroy, 1985). However, identification of 1-hydroxypyrene is the first direct identification of cytochrome P450-like PAH metabolism in *Arenicola marina*. Previous studies have failed to detect pyrene metabolites (Christensen et al., 2002) or a PAH metabolizing system in *Arenicola marina* (Payne & May, 1979), though arenicolids are known to excrete aqueous metabolites of pyrene and other PAHs (Christensen et al., 2002; Kure, 1997; Weston, 1990).

By applying the tentative identification scheme to all four species (visualized by the bands lines in Fig. 6) distinct differences in pyrene metabolism, especially in

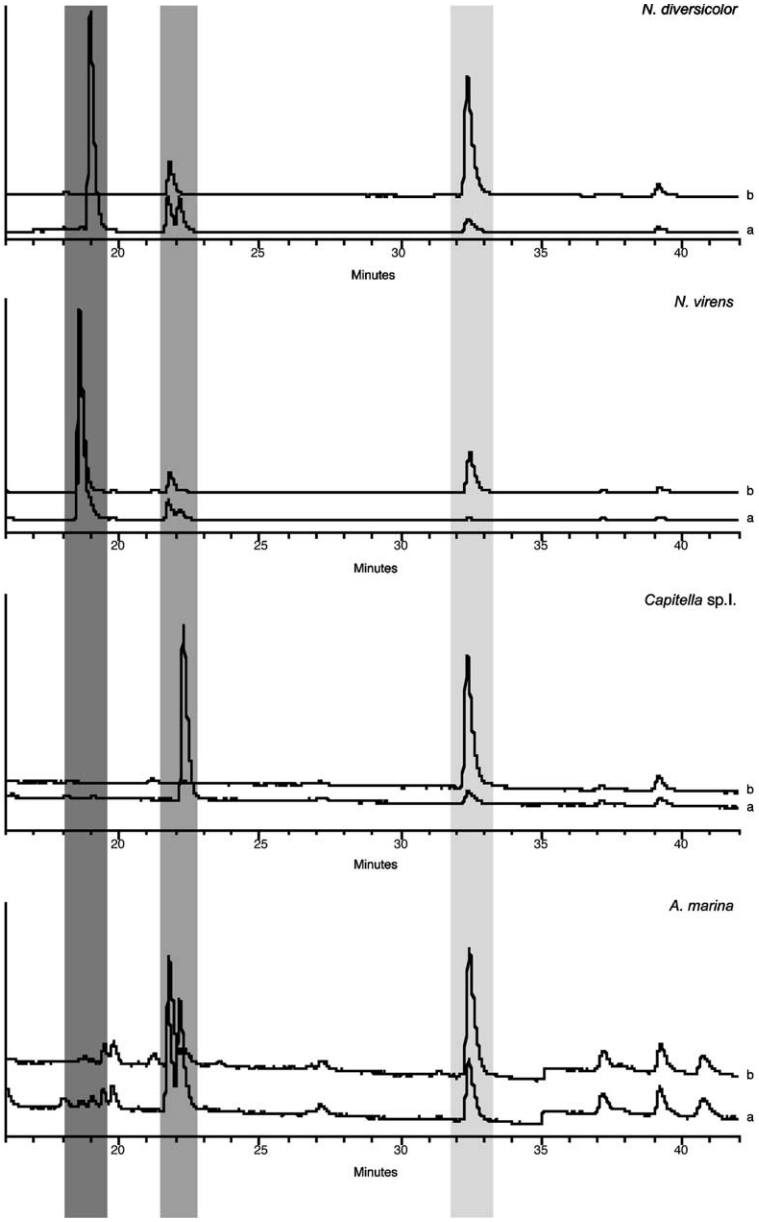

Fig. 6. Fluorescence chromatograms of tissue samples presented in Fig. 5. Lower chromatogram (marked *a*) for each species is the sample prior to enzymatic hydrolysis and the upper (marked *b*) shows the effect of enzymatic hydrolysis. 1-Hydroxypyrene appears at 32 mm (light gray band) in tissue from all four species and the peak increases in size after treatment with glucuronidase-arylsulfatase. The traces (*a* and *b*) are offset and abscissa is limited to metabolite time window for visualization purposes. The darker gray band to the left is 1-hydroxypyrene glucuronide and the gray band in the middle contains 1-hydroxypyrene sulfate and 1-hydroxypyrene glucoside peaks.

conjugated metabolites, are observed among the four species. The tentative phase II metabolite identification above suggests that nereid polychaetes predominantly make use of glucuronide conjugation whereas *Capitella* sp. I. and *Arenicola marina* appears to utilize predominantly sulfate and/or glucoside conjugation respectively. An interesting question becomes whether an advantage is gained by using either of these strategies. Deposit-feeding polychaetes like *Capitella* sp. I. often dominate oil-contaminated sediments (Grassle & Grassle, 1974; Grassle & Grassle, 1976; Pearson & Rosenberg, 1978) whereas *Arenicola marina* is known to be more sensitive to PAH contamination (Kure, 1997). The gut of a deposit-feeder is assumed to be the primary route of both uptake and elimination of organic contaminants in deposit-feeding polychaetes (Forbes, Forbes, Giessing, Hansen, & Kure, 1998; Mayer et al., 1996) and elimination of aqueous metabolites through gut fluid is expected as an aqueous intermediary necessary for excretion (Giessing et al., 2003). Assuming that our tentative identification of phase II metabolites in *Arenicola marina* is correct, hydrolysis of 1-hydroxypyrene glucoside by β-glucosidase enzymes present in gut fluid of most deposit-feeders (Mayer et al., 1997) would release 1-hydroxypyrene which would potentially reabsorbed into the animal thereby increasing toxicity. A similar process, called entero-hepatic circulation, has been observed in vertebrate as well as invertebrate species (James, 1987).

Synchronous fluorescence spectroscopy of whole worm homogenates is a straightforward, fast, and simple method for the detection of pyrene metabolites and assessment of PAH exposure in marine, deposit-feeding polychaetes. The complete analysis takes less than 5 min. Even though the present study suggests that HPLC/UV/F surpasses SFS as a quantitative analytical technique for pyrene and its metabolites, SFS can provide a useful and rapid method and could be a good screening tool for PAH exposure in marine invertebrates. Detection of 1-hydroxypyrene can indicate accumulation of pyrene, integrated over all uptake routes, and taking into account bioavailabiity. Activities of specific enzymes such as cytochrome P450 and 7-ethoxyresorufln-*O*-deethylase (EROD) can be used as biochemical markers of environmental contamination. However, these enzymes can be induced by multiple substrates (e.g. PAHs, dioxins and PCBs) and no detection of pyrene metabolites at zero exposure makes the SFS method more specific to PAH exposure.

The use of 1-hydroxypyrene as a biomarker for total PAH exposure has successfully been applied to fish bile. Field studies have indicated that the sensitivity of the SFS method is sufficient for field monitoring close to industrialized and urbanized areas (Ariese et al., 1993c; Krahn, Burrows, MacLeod, & Malins, 1987; Van der Oost, Van Schooten, Ariese, Heida, Satumalay, & Vermeulen, 1994). Furthermore, studies have shown that the total PAH metabolite profile in fish bile is roughly constant at different locations with comparable PAH sources, and that l-hydroxypyrene, easily quantified by SFS, can be used as a relative measure for the total uptake of PAHs (Ariese et al., 1993b; Krahn et al., 1987; Lin et al., 1994). Future experiments will reveal if a similar correlation between total PAH and 1-hydroxypyrene can be made in marine invertebrates in both laboratory and field experiments.

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