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1 1-Hydroxypyrene as a biomarker of PAH exposure in the marine
2 polychaete *Nereis diversicolor*

3
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10
11 **Abstract**

12 The possibility of using the pyrene metabolite 1-hydroxypyrene as a biomarker of
13 polycyclic aromatic hydrocarbons (PAH) exposure was investigated by exposure of the marine
14 polychaete *Nereis diversicolor* to several PAHs in the laboratory. Animals were exposed to pyrene
15 alone and to five different PAHs - phenanthrene, anthracene, pyrene, benzo[a]pyrene and
16 benzo[k]fluoranthene. After 5 days of exposure the concentrations of parent PAHs and 1-
17 hydroxypyrene were identified using three different analytical methods, high-performance liquid
18 chromatography with fluorescence detection (HPLC/F), synchronous fluorescence spectroscopy
19 (SFS) and gas chromatography with mass spectrometric detection (GC/MS). The SFS
20 measurements of 1-hydroxypyrene were validated by the more sensitive method HPLC/F. The
21 positive correlation between total PAHs and 1-hydroxypyrene concentrations in the polychaete
22 tissues observed in experiments, suggests the feasibility of 1-hydroxypyrene as a suitable
23 biomarker for total PAH exposure assessment. Furthermore, the possibility of employment of the
24 simple and rapid SFS method instead of HPLC/F for biomarker analysis has been confirmed by
25 the positive and significant correlation between results achieved by these two analytical methods.

26
27 **Keywords:** 1-Hydroxypyrene; *Nereis diversicolor*; Biomarker; Polychaete; PAH; HPLC; SFS;
28 Sediment pollution.

29
30 **1. Introduction**

31 Environmental contamination with polycyclic aromatic hydrocarbons (PAHs) is a matter
32 of concern, as this contamination is widespread in both marine and terrestrial environments and
33 some PAHs are carcinogenic (Neff, 1985; Ariese et al., 1993; Giessing et al., 2003a). PAH
34 pollution originates from both natural and anthropogenic sources, the latter being the major
35 contributor (Levin, 1995). Being natural components of fossil fuels, PAHs are emitted to the
36 environment due to incomplete combustion and as a result of crude oil spillages (Walker et al.,
37 2001). Generally, environmental exposure of organisms to PAHs is assessed by monitoring their
38 environment (sediment/soil, water, and air), and routine monitoring of contaminants levels in the
39 aquatic environment usually involves determination of parent PAH in sediment and animal tissue
40 samples (Ariese et al., 1993; Fillmann et al., 2004). However, chemical analysis of these samples
41 provides little useful information for compounds that are readily metabolized and, therefore,
42 biomonitoring of PAH exposure should include determination of PAH metabolites in animal
43 tissues.

44 Pyrene is a constituent of all environmental mixtures of PAH (Levin, 1995; Jongeneelen et
45 al., 1985). Pyrene is not particularly toxic and only one phase I metabolite, 1-hydroxypyrene, is
46 formed by biotransformation process in eukaryotic organisms (Giessing et al., 2003a). As this is
47 an extensive analytical advantage, pyrene is a widely used model compound in studies of PAH

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48 metabolism (Giessing et al., 2003a). Being a major metabolite of a representative compound in
49 PAH mixtures, 1-hydroxypyrene can be used as a biomarker of total PAH exposure (Levin, 1995).

50 1-Hydroxypyrene was introduced as a biomarker of human occupational PAH exposure by
51 Jongeneelen et al. (Jongeneelen et al., 1985). Its applicability as a biomarker in humans has been
52 tested and verified in hundreds of studies (Levin, 1995; Jongeneelen et al., 1987; Jongeneelen et
53 al., 1988; Goen et al., 1995; Boogaard and Vansittert, 1995; Tsai et al., 2002; Tsai et al., 2004;
54 Levin et al., 1995; Strickland and Kang, 1999; Viau et al., 1995; Viau and Vyskocil, 1995). Based
55 on a robust and non-laborious analytical method urinary 1-hydroxypyrene is a useful biomarker of
56 human PAH exposure (Jongeneelen et al., 2001). In fish, PAH exposure can be assessed by
57 screening the gall bladder bile for 1-hydroxypyrene (Ariese et al., 1993; Aas et al, 1998). Also in
58 several invertebrate species, e.g. Dungeness crab and the terrestrial isopod *Porcellio scaber*, 1-
59 hydroxypyrene has been used as a suitable indicator for PAH exposure (Eickhoff et al, 2003;
60 Stroomberg et al., 1999). In the present study we extend the use of 1-hydroxypyrene as biomarker
61 of environmental PAH exposure to marine sediments, by use of the sessile infaunal invertebrate
62 species of marine polychaete *Nereis diversicolor*.

63 Analytical methods for identification and quantification of PAHs and their metabolites
64 usually involve chromatographic separation by high-performance liquid chromatography (HPLC)
65 or gas chromatography (GC) with mass spectrometric or fluorescence detection (Ariese et al.,
66 1993; Lin et al., 1994). The total analysis time using these methods is considerable in comparison
67 to fluorescence spectroscopy methods. Environmental monitoring often requires screening large
68 numbers of samples and, therefore, it is desirable to use a simpler, less expensive and time
69 consuming method to assess PAH exposure than, for example, gas chromatography with mass
70 spectrometric detection GC/MS or high-performance liquid chromatography with fluorescence
71 detection HPLC/F (Ariese et al., 1993; Lin et al., 1994). However, more rapid alternative
72 analytical methods for biomonitoring must be just as sensitive and specific for PAHs as
73 chromatographic methods.

74 Conventional fluorescence spectroscopy has an excellent sensitivity in determining trace
75 organic compounds but its application to simultaneous determination of several fluorescent
76 compounds in mixtures is limited due to spectral overlap for compounds with high structural
77 similarity (Rodriquez and Sanz, 2000), as is the case for pyrene and 1-hydroxypyrene. Such
78 spectral interferences can, sometimes, be resolved by synchronous fluorescence spectroscopy
79 (SFS), i.e. by scanning both the excitation and emission simultaneously with a constant
80 wavelength difference (Eickhoff et al, 2003; Lin et al., 1994; Giessing et al., 2003b). In recent
81 years, SFS has been used increasingly to screen for pyrene metabolites in various matrices
82 including human urine (Strickland et al., 1994), fish bile (Ariese et al., 1993; Lin et al., 1994), and
83 crab hemolymph and urine (Eickhoff et al, 2003; Watson et al., 2004). Giessing et al. (2003)
84 showed the usefulness of 1-hydroxypyrene measured by SFS as a biomarker for pyrene exposure
85 in *Nereis diversicolor*, demonstrating a good correlation between SFS and HPLC with ultraviolet
86 detection (HPLC/UV) measurements of 1-hydroxypyrene (Giessing et al., 2003b). In the present
87 study the formation of pyrene metabolites in the marine polychaete *Nereis diversicolor* was
88 investigated, with special attention given to the detection of 1-hydroxypyrene using SFS. HPLC/F
89 and GC/MS were used as complementary methods for confirmation of the SFS results.

90 To evaluate the feasibility of 1-hydroxypyrene as biomarker for assessing environmental
91 exposure to PAHs, the correlation between 1-hydroxypyrene and total PAH content in animal
92 tissues and sediment was investigated in experiments, where *Nereis diversicolor* was exposed to
93 mixture of different PAHs. Accordingly, the objectives of this study were to evaluate the
94 feasibility of (i) 1-hydroxypyrene in polychaete worms as a biomarker for assessing total PAH
95 exposure, and (ii) a rapid SFS method for identification and quantification of 1-hydroxypyrene.

96

97 2. Materials and Methods

98 2.1 Chemicals

99 Pyrene (98%) and 1-hydroxypyrene (98%) were purchased from Sigma Aldrich (Germany). EPA
100 Polynuclear Aromatic Hydrocarbons Kit 610 - N (phenanthrene (99,9%), anthracene (99,1%),
101 pyrene (98%), benzo[a]pyrene (96,9%) and benzo[k]flouranthene (99,7%)); EPA 610 Polynuclear
102 Aromatic Hydrocarbons Mix, solution in methanol:methylene chloride (1:1); Quebec Ministry of
103 Environment PAH MIX in methylene chloride:benzene (75:25), were purchased from Supelco
104 (USA). HPLC grade acetone, acetonitrile, methanol were acquired from Lab Scan (Dublin,
105 Ireland). Ethanol (96%) from De Danske Spritfabrikker A/S (Denmark). Enzyme β -glucuronidase-
106 arylsulfatase (from *Helix pomatia*, 108,750 U/ml, and 134,3 U/ml) was supplied by Sigma Aldrich
107 (Germany). Ammonium acetate and Acetic acid (100%) were from Merck (Germany). All
108 chemicals were used as received.

109 2.2 Laboratory studies

110 2.2.1 Test animals. Individual *Nereis diversicolor* were collected on a sand flat at Store Havelse,
111 Roskilde Fjord, Denmark. Worms were sorted according to size and physical condition in order to
112 use a homogeneous collection of animals for experiments. Animals were kept in culture in native
113 sediment with seawater (16 ‰) from the fjord for a minimum of 7 days prior to use.
114

115 2.2.2 Sediment spiking. Sediment collected at the same time as worms was sieved (1 mm) and
116 frozen (-18°C) until use. Thawed sediment was contaminated by adding the desired amounts of
117 PAHs dissolved in a minimum amount of acetone to sediment slurry and 0.45 μ m filtered
118 seawater. This slurry was mixed continuously with a pillar drilling machine for approximately 12
119 hours. After settling, overlying water was decanted, and the sediment was kept at 4°C for 1 week
120 prior to use in microcosms. In this way sediments with different concentrations of pyrene (pyrene
121 exposure experiment) or of a mixture of 5 PAHs - phenanthrene, anthracene, pyrene,
122 benzo[a]pyrene (B[a]p) and benzo[k]flouranthene (B[k]f) (five PAHs experiment) were produced.
123

124 2.2.3 Concentration-response relationship experiments with pyrene. Three independent replicates
125 in plastic containers with 200 ml of sediment and 200-300 ml of overlying filtered seawater water
126 (16 ‰) were established at 1, 10 and 30 mg pyrene kg^{-1} sediment (dw). Each container was
127 continuously aerated throughout the experiment. Individuals of *Nereis diversicolor* were added (1
128 worm per container). After 5 days of exposure whole worms were collected and treated as
129 described below. Pyrene and 1-hydroxypyrene were identified and quantified using HPLC/F and
130 SFS.
131

132 2.2.4 Five PAHs exposure experiment. Three independent replicates in plastic containers with 200
133 ml of sediment and 400 ml of overlying filtered seawater (16 ‰) were established at nominal
134 concentrations: 2.5, 10 and 50 mg of total PAH kg^{-1} sediment (dw). The total PAH concentration
135 is the sum of the concentrations of all five compounds: phenanthrene, anthracene, pyrene, B[a]p,
136 B[k]f, in equal weight proportions. Each container for the exposure concentrations 2.5 and 10 mg
137 kg^{-1} sediment (dw) - contained five worms, while the concentration 50 mg kg^{-1} sediment (dw) and
138 control treatments contained one worm in each replicate. Each container was continuously aerated
139 throughout the experiment. After 5 days of exposure whole worms were collected and treated as
140 described below. Phenanthrene, anthracene, pyrene, b[a]p and b[k]f were identified and quantified
141 using HPLC/F. The amount of 1-hydroxypyrene was measured using HPLC/F and SFS.
142

143 2.3 Sample treatment

144 Worms were collected after 5 days of exposure and allowed to defecate in individual aluminum
145 dishes containing overlying water from the experimental container. After four hours most of the
146 worms had purged their guts of the sediment (visual observation). The worms were weighed (ww)
147

148 and transferred to test tubes containing 2 ml of methanol and, subsequently, homogenized using a
149 Tissue Tearor (Biospec Products, Inc. Bartlesville, OK, USA) at full speed for 1 min. Samples
150 were then sonicated for 10 min and, subsequently, centrifuged at 1239 g for 10 min to precipitate
151 debris. The supernatant was collected and filtered through a 0.22 μm syringe filter and transferred
152 directly to brown HPLC vials without any further sample preparation for the pyrene single
153 exposure experiment. For the five PAHs experiment, samples from the two lowest exposure
154 concentrations were pooled in the following way: five animals from the same container were
155 extracted separately in methanol. Upon centrifugation, supernatants were pooled into one vial.
156 Pooled samples were then evaporated to dryness under a gentle flow of nitrogen and re-diluted in
157 2 ml of methanol. All samples were stored at -80°C prior to further treatments.

158
159 *2.3.1 Enzymatic hydrolysis.* Glucuronidase-arylsulfatase enzymes hydrolyze glucuronide and
160 sulfate conjugates to give free 1-hydroxypyrene (Giessing et al., 2003b). To achieve maximum
161 enzymatic hydrolysis and get an amount of 1-hydroxypyrene per sample sufficient for SFS
162 analysis, the concentration of tissue extracts in methanol, amount of enzyme units, and time of
163 incubation at 37°C were optimized. The enzyme activity is inhibited by the presence of methanol
164 in the extracts. Thus, since the amount of the sample incubated with enzyme was relatively high
165 (up to 1:10 final dilution) we added more enzyme and applied longer incubation period than
166 previously described (Giessing et al., 2003b).

167 Tissue methanol extracts from the pyrene exposure experiment were diluted 1:3 in
168 ammonium acetate buffer (pH=5) and diluted 1:150 with 0.22 μm filtered deionized water (DI) and
169 incubated for 18 hrs at 37°C with approximately 100 units of β -glucuronidase-arylsulfatase. After
170 addition of 500 μl of ice-cold 99.9% ethanol, samples were mixed and centrifuged at 3000 g for 5
171 min at 4°C to precipitate denatured protein.

172 Tissue methanol extracts from the five PAHs experiment were diluted 1:25 with 0.22 μm
173 filtered DI and incubated for 22 hrs at 37°C with approximately 100 units of β -glucuronidase-
174 arylsulfatase. After addition of 500 μl of ice-cold 99.9% ethanol, samples were mixed and
175 centrifuged at 3000 g for 5 min at 4°C to precipitate denatured protein.

176 The supernatant from both treatments was transferred to brown HPLC vials and analyzed
177 using HPLC/F and SFS.

178
179 *2.3.2 Synchronous fluorescence spectroscopy.* Samples from enzymatic hydrolysis were analyzed
180 by SFS (Perkin Elmer Luminiscence spectrometer LS50B) without further treatment, using a 5
181 mm quartz cuvette. The spectroscopic conditions were set as 5 nm slits on excitation and emission
182 channels and photomultiplier tube (PMT) voltage of 900 V, using a scan speed of 1200 nm/min,
183 and scanning the wavelength range 250 - 400 nm with a constant wavelength difference of 34 nm
184 between excitation and emission channels. Calibration curves were made by preparing standard
185 dilutions of 1-hydroxypyrene in methanol added into a tissue matrix from extracts of unexposed
186 worms. For the analysis of samples from the pyrene single exposure experiment, the standard
187 dilutions of 1-hydroxypyrene were added into tissue matrix without treating it with enzyme. For
188 the quantitative analysis of samples from the five PAHs exposure experiment, standard dilutions
189 of 1-hydroxypyrene were added into matrix, tissue of unexposed worm, which had undergone the
190 same treatment with enzyme as the analytes. Calibration curves had regression coefficients $r^2 >$
191 0.98 in both cases.

192
193 *2.3.3 High performance liquid chromatography.* Quantification of metabolites and parent PAHs
194 was done by HPLC/F. The system consisted of a Dionex P680 HPLC Pump (2002 Dionex,
195 Version 1.0), an automated Sample Injector ASI-100 (2001 Dionex Softron GmbH, Version 1.14),
196 a column oven STH585 (2000 Dionex Softron GmbH, Version 2.50) and a Dionex RF2000
197 Fluorescence Detector (2000 Dionex Softron GmbH, Version 1.40).

198 Quantification of pyrene and 1-hydroxypyrene in tissue samples from *Nereis diversicolor*
199 was done using a Phenomenex Synergi 4u Hydro-RP 80A C18 column, 15 cm x 4.6 mm
200 (Phenomenex, USA). The acetonitrile/water gradient profile was 50:50 for 5 min, 100:0 over 10
201 min, held for 5 min, and finally 50:50 over 5 min at a flow rate of 1 ml min⁻¹. Column temperature
202 was kept at 28°C. The excitation and emission wavelengths of the fluorescence detector were set
203 at 346 nm and 384 nm, respectively. The excitation wavelength was changed to 333 nm after 14
204 min. Spectral bandwidths of the fluorescence detector were always fixed at 15 nm for both,
205 excitation and emission.

206 Identification of conjugated metabolites and 1-hydroxypyrene in tissues of *Nereis*
207 *diversicolor* prior to enzymatic treatment was performed using a reversed phase Phenomenex
208 Synergi 4u Fusion-RP 80A C18 column, 25 cm x 4.6 mm (Phenomenex, USA). The
209 acetonitrile/buffer (10 mM ammonium acetate buffer, pH=5) gradient profile used for the
210 quantification of conjugated metabolites and 1-hydroxypyrene was 5:95 for 2 min, the proportion
211 of acetonitrile increased linearly over 35 min to 90:10, held for 13 min, and decreased to 5:95 over
212 10 min at a flow rate of 0.8 ml min⁻¹. Column temperature was kept at 28°C. The excitation and
213 emission wavelengths of the fluorescence detector were set at 346 nm and 384 nm, respectively,
214 to measure metabolites, and then the excitation wavelength was changed to 333 nm after 37 min,
215 to measure the parent compound.

216 Quantification of phenanthrene, anthracene, pyrene, benzo[a]pyrene and
217 benzo[k]fluoranthene was carried out, using a Primshere C18 column (25 cm x 4.6 mm,
218 Phenomenex, Torrance, CA, USA). The acetonitrile/water gradient was 40:60 for 2 min, then
219 increased at a linear rate to 100:0 over 20 min, held for 8 min, and changed back to 40:60 over 2
220 min, at a flow rate of 1.5 ml min⁻¹. The column temperature was kept at 30°C. The excitation and
221 emission wavelengths of the fluorescence detector were set at 254 nm and 395 nm, respectively.
222 During the run, the excitation/emission wavelengths were changed twice: to 335 nm/380 nm after
223 19 min and to 290 nm/420 nm after 22 min.

224 The identification and quantification of 1-hydroxypyrene and parent PAHs was performed
225 using external standard dilutions of these compounds in methanol.

226
227 *2.3.4 Tissue samples for GC/MS analysis.* Tissue extracts for total PAH content analysis by
228 GC/MS were prepared in two steps. The first extraction step was with methanol as previously
229 described for HPLC/F analysis. After removing the methanol phase, tissues were re-extracted in
230 dichloromethane (DCM) to extract residual PAH from animal tissues. 2 ml of DCM were added
231 and the procedure was then as described for methanol extraction. The DCM phase of tissue
232 extracts was collected upon centrifugation and samples were dried using Na₂SO₄ containing glass
233 pipettes. Aliquots were evaporated down to 1 ml under a gentle flow of nitrogen, prior to analysis
234 on GC/MS.

235
236 *2.3.5 Sediment samples GC/MS analysis.* Wet sediment samples collected at the end of the
237 experiment were extracted in 30 ml of DCM in a Microwave Accelerated Reaction System
238 (Model MARS 5®; CEM Corporation) by ramping for 10 min. to reach 110°C and then holding
239 for 25 min at this temperature. Cooled samples were dried using Na₂SO₄ and cleaned of organic
240 debris using columns filled with activated Silica gel and Na₂SO₄. Columns were eluted with 40 ml
241 DCM and extracts were evaporated to lower their volume under a gentle flow of nitrogen. Extracts
242 were dried again using Na₂SO₄ prior to GC/MS analysis. Internal and recovery standards, both
243 containing deuterated PAHs, were added to the extracts at the beginning and at the end of the
244 extraction procedure, respectively. External calibration curves were used for the quantification
245 analysis of each of the PAHs. All calibration curves had regression coefficients $r^2 > 0.98$.

246

247 **2.3.6 Gas chromatography mass spectrometry.** Gas chromatography mass spectrometric (GC/MS)
248 analysis of sediment and tissue samples from the five PAHs experiment was performed on a
249 Finnigan TRACE DSQ™ Single Quadrupole GC-MS (Thermo Electron Corporation) with a 60 m
250 HP-5MS capillary column (0.25 mm i.d. x 0.25 μ m film) and operated in electron ionisation (EI)
251 mode. The injection volume was 1 μ l in programmable temperature vaporization (PTV) inlet,
252 Solvent Split mode. Starting (base) temperature: 65°C; solvent valve temperature: 100°C;
253 evaporation rate 1°C/sec; evaporation temperature 70°C; transfer rate 14.5 °C/s; temperature was
254 held for 1 min at 290°C during transfer; carrier gas: helium (1.0 ml/min); oven program: 40°C
255 (1min), 40°C/min to 80°C, 15°C/min to 160°C, 5°C/min to 220°C, 15°C/min to 315°C (10min).
256 Data acquisition time 4.3 scans/s. Mass spectrometric conditions: transfer liner and ion source
257 temperature: 300°C and 250°C, respectively. Eight mass fragments were analyzed in 2 groups of 4
258 ions using SIM. The commercial GC-MS software *Xcalibur 1.3* was used for data processing.

259

260 **2.4 Statistical analysis**

261 Correlation analysis was performed using Spearman's Rank-order test.

262

263 **3. Results and Discussion**

264

265 **3.1 Synchronous fluorescence spectrometry**

266 Synchronous fluorescence spectrometry (SFS) analysis of the tissue extracts of the worms
267 exposed to pyrene revealed peaks representing 1-hydroxypyrene and conjugated pyrene
268 metabolites (Fig. 1). The peak representing conjugates is blue shifted in relation to the 1-
269 hydroxypyrene peak and has a maximum intensity at an excitation wavelength of 346 nm (Fig. 1
270 A v). The blue shift of conjugated 1-hydroxypyrene is in accordance with previous studies (Ariese
271 et al., 1993; Giessing et al., 2003b). Although Ariese et al. (1993) reported increase in
272 fluorescence intensity of glucuronide conjugated 1-hydroxypyrene 2-fold, and Strickland et al.
273 (1994) observed 5-fold higher intensity in 1-hydroxypyrene glucuronide than in free 1-
274 hydroxypyrene, there was only a minor intensity increase in glucuronide conjugate of 1-
275 hydroxypyrene, in our study (Fig.1). As expected, conjugated metabolites were not detectable in
276 the tissue sample after glucuronidase treatment except for pyrene-1-sulfate that remained in the
277 sample in minor quantities. 1-hydroxypyrene was the only peak present in the spectrum after
278 enzymatic hydrolysis and had its maximum fluorescence in the SFS spectrum at 351 nm excitation
279 wavelength (Fig. 1 A iv). The wavelength of maximum fluorescence for 1-hydroxypyrene is in
280 accordance with previous studies, where the distinctive excitation maxima of 1-hydroxypyrene
281 were at 352 nm (Strickland et al., 1994).

282

283 Fig. 1.

284

285 The maximum fluorescence intensity for the pyrene peak in the reference standard sample
286 was at 336 nm (Fig. 1 A ii). However, pyrene was never detected in the tissue samples using SFS
287 or HPLC/F, after enzymatic treatment of the sample. Pyrene was below the detection level in
288 these samples, probably due to precipitation with the enzyme and other organic matter during the
289 enzymatic hydrolysis procedure.

290 An overlay of spectra of the tissue samples from the worms exposed to five different
291 PAHs, before and after enzymatic treatment is shown in Fig. 1 B, together with the spectra of
292 unexposed worms from the same experiment and 1-hydroxypyrene reference standard solution
293 (same as in Fig. 1 A). The conjugated metabolite peak is, as well as in the pyrene exposure
294 experiment, blue shifted in relation to 1-hydroxypyrene, having its maximum fluorescence at
295 346.9 nm (Fig. 1 B iv).

296 The first broad band at around 280 nm present in all samples (Fig. 1 A, Fig. 1 B)
297 represents traces of protein-lipid matrix, which cannot be completely removed after precipitation

298 by ice-cold ethanol and centrifugation. Spectra from unexposed worms (Fig. 1 A i, Fig. 1 B i)
299 showed no peaks other than this broad protein band with varying intensity, in agreement with a
300 previous study (Giessing et al., 2003b).

301 The main feature of both figures (Fig. 1 A and Fig. 1 B) is spectral similarity. After
302 preparative treatment, tissue extracts from organisms exposed to several different PAHs reveal
303 similar characteristics as samples from animals exposed only to pyrene. Evidently from the Fig. 1
304 B, no other fluorescence is interfering with the 1-hydroxypyrene peak in the spectra. This fact
305 clearly demonstrates the possibility of measuring a proposed biomarker compound - 1-
306 hydroxypyrene, in tissue samples from organisms exposed to complex mixtures of multiple PAHs.

307 **3.2 High performance liquid chromatography**

308 To validate fluorometric measurements in relation to possible interferences of different
309 non target compounds the SFS method for screening for PAH contamination was verified using
310 HPLC, and the samples were enzymatically hydrolyzed prior to quantification (Giessing et al.,
311 2003b). The conjugated metabolites, 1-hydroxypyrene and unchanged pyrene in the tissues were
312 identified using HPLC/F. The conjugated metabolites were identified according to the order of
313 elution as established in the method developed in our laboratory by Jørgensen et al. (2005). A
314 typical HPLC/F chromatogram of the tissue extract shows five peaks representing: 1-
315 hydroxypyrene glucuronide (retention time = 16.2 min), 1-hydroxypyrene sulfate (retention time =
316 18.86 min) and 1-hydroxypyrene glucoside (retention time = 22.46 min). The 1-hydroxypyrene
317 peak elutes later than the conjugated metabolites and the unmetabolized pyrene peak is the last
318 one in the chromatogram (retention time = 37.3 min) (Fig. 2). Pretreatment of the tissue extracts
319 with β -glucuronidase-arylsulfatase reduced or eliminated the conjugated metabolite peaks with a
320 concomitant increase in the 1-hydroxypyrene peak (retention time = 31.62 min). It was possible to
321 eliminate 1-hydroxypyrene glucuronide and 1-hydroxypyrene glucoside metabolites but the
322 sulfate conjugate peak could not be sufficiently hydrolysed, thus the peak was only reduced in
323 size.
324

325
326 Fig. 2.

327
328 As fluorescence efficiencies are higher for the conjugates than for 1-hydroxypyrene, the
329 amount of pyrene conjugates can be expressed in 1-hydroxypyrene equivalents, by adjusting the
330 pyrene conjugate area with conversion factors (Stroomberg et al., 2003). In this study the peak
331 areas of pyrene-1-glucuronide, pyrene-1-glucoside and pyrene-1-sulfate were divided by factors
332 1.8, 1.8 and 1.7 respectively (Jørgensen, personal communication).

333 The amount of remaining conjugates in the tissues of animals exposed to the five PAHs is
334 higher than in animals exposed to pyrene only. Animals exposed to five different PAHs produce
335 many other conjugated metabolites than those of pyrene, hence, competition of substrates for the
336 enzyme is possible, and increased amounts of enzyme should be added.

337 Complete hydrolysis of the conjugated 1-hydroxypyrene was not an obligatory condition
338 for this study, as there were no noticeable spectral interferences for the SFS analysis that could be
339 caused by conjugated metabolites after deconjugation with enzymatic hydrolysis. The amount of
340 remaining conjugates was insignificant for the reproducibility of the measurement, thus the
341 method was not affected by incomplete hydrolysis.

342 SFS analysis of 1-hydroxypyrene and its conjugates was carried out using $\Delta\lambda$ of 34
343 (Giessing et al., 2003b; Lin et al., 1994) and 37 nm (Ariese et al., 1993) in different studies. Here,
344 the SFS spectra were recorded with a wavelength interval $\Delta\lambda$ of 34 nm.

345 A HPLC/F procedure for detection of PAH metabolites in fish bile was developed by
346 Krahn et al. (1984) as a relatively inexpensive and rapid screening method, which does not require
347 complete separation or identification of individual metabolites, as it was a rapid separation of non-

348 hydrolyzed bile sample by reverse-phase HPLC, and the overall fluorescence over a specific
349 elution interval was integrated to give a sum parameter that is expressed as the amount of
350 “equivalent” to the chosen standard compound (Ariese et al., 2005). Furthermore, HPLC/F
351 showed to be useful for accurate measure of individual deconjugated PAH metabolites in
352 enzymatically hydrolyzed bile samples (Ariese et al., 2005). Thus, the HPLC/F method has been
353 shown to be sensitive and reliable for analysis of PAH metabolites in different matrices, such as
354 fish bile (Ariese et al., 1993; Lin et al., 1994; Krahn et al., 1984; Krahn et al., 1986) and marine
355 and terrestrial invertebrates tissues (Stroomberg et al., 1999; Giessing et al., 2003b; Stroomberg et
356 al., 2003). However, the SFS method has its advantages such as simplicity and speed, and it also
357 requires less instrumentation and maintenance than HPLC/F. Results represented in figures 1 and
358 2 clearly demonstrate, that SFS could be a possible substitution method instead of HPLC/F for
359 analysis of 1-hydroxypyrene, since it has sufficiently high sensitivity for this compound, and
360 separation of 1-hydroxypyrene from other PAH compounds and metabolites is not necessary.

361

362 **3.3 Using 1-hydroxypyrene concentrations to characterize PAH exposure**

363 In the concentration-response relationship experiment, worms exposed to pyrene showed
364 increasing accumulation of pyrene and production of 1-hydroxypyrene with increasing exposure
365 concentration (Fig. 3). The exposure period was 5 days, because a pyrene accumulation study by
366 Christensen et al. (2002) showed that pyrene accumulation reached a steady-state in the tissues of
367 *N. diversicolor* within 4-5 days (Christensen et al., 2002).

368 These results, increasing tissue accumulation of pyrene and production of 1-
369 hydroxypyrene with increasing exposure concentration, agree with those of a previous study of
370 Giessing et al. (2003b). This observation indicates usefulness of this metabolite as a biomarker of
371 pyrene exposure. The concentrations of both 1-hydroxypyrene and pyrene were below the
372 detection limit in samples of unexposed worms. Pyrene was not detectable by HPLC/F in tissue
373 samples after enzymatic hydrolysis, probably due to precipitation with organic matter during the
374 enzymatic hydrolysis procedure, as is the case of SFS measurements. This is in agreement with
375 the study of Giessing and Mayer (2004) who observed coprecipitation of pyrene with the matrix
376 components after the centrifugation.

377

378 Fig. 3.

379

380 Tissue concentrations of 1-hydroxypyrene from the pyrene only exposure experiment were
381 quantified using SFS and HPLC/F. In samples from the lowest exposure concentration: 1 mg
382 pyrene kg⁻¹ sediment (dw), 1-hydroxypyrene was below the detection limit when using SFS,
383 however, it was possible to measure 1-hydroxypyrene at the higher exposure concentrations: 10
384 and 30 mg pyrene kg⁻¹ sediment (dw). A positive and highly significant correlation was observed
385 (Spearman's rank-order correlation, $r_s = 0.903$; $P < 0.01$) between 1-hydroxypyrene concentrations
386 measured by the two methods (Fig.4). These results are a fine confirmation of the previous results
387 in similar experiment in the study of Giessing et al. (2003b).

388

389 Fig. 4.

390

391 The 1-hydroxypyrene concentrations measured by SFS are on average 1.4 times higher
392 than those measured by HPLC/F with all but two of the data points above the 1:1 line on Fig.4.
393 Conjugated 1-hydroxypyrene has higher fluorescence intensity than the free metabolite (Ariese et
394 al., 1993; Strickland et al., 1994). As mentioned earlier, in the absence of enzymatic hydrolysis,
395 the SFS signal measured is the combined signal of all compounds containing the 1-hydroxypyrene
396 fluorophore. Thus, 1-hydroxypyrene concentrations determined by SFS would be expected to be
397 higher than those determined by HPLC/F (Giessing et al., 2003b). Although we have employed
398 enzymatic hydrolysis in our study, it was not possible to achieve 100% complete hydrolysis of

399 conjugates. Consequently, a fraction of conjugates still remained in the samples, adding to the
400 fluorescence signal of the 1-hydroxypyrene containing fluorophore. Thus, 1-hydroxypyrene
401 concentrations determined by SFS, being sum of all 1-hydroxypyrene containing species, would
402 be expected to be higher than concentrations determined by HPLC also in our study. Another
403 explanation for the difference may be the way the calibration solutions were prepared. As
404 described in the Materials and Methods, the standard dilutions for SFS analysis contained protein-
405 lipid matrix in amounts equal to those in the samples. If standard dilutions were prepared
406 separately from samples, differences in preparation conditions may develop, that could result in
407 varying degree of precipitation of the protein-lipid matrix. Consequently, an elevated baseline in
408 SFS spectra of the standard dilutions could lead to a slight increase in the 1-hydroxypyrene
409 concentrations measured. Thus, for our further investigations, we prepared the matrix solutions for
410 SFS calibration curves at the same time as the tissue samples of exposed worms and in precisely
411 the same manner.

412 Giessing et al. (2003b) first reported the use of SFS analysis for measuring pyrene
413 metabolites in *N. diversicolor* exposed to pyrene, as a substitute to HPLC/F analysis, and studied
414 the correlation of results derived by these two methods. When comparing our correlation results to
415 those of Giessing et al. (2003b), some differences in correlation strength, as well as values
416 achieved by SFS measurement in contrast to values derived by HPLC/F are noticeable. In the
417 present study the SFS values differ less from the HPLC/F values, because the enzymatic
418 hydrolysis was applied to the samples. The correlation is also stronger in our study with $P < 0.01$
419 compared to $P = 0.081$ of Giessing et al. (2003b).

420 In light of the complex composition of environmental PAH mixtures, it is necessary to
421 examine the following before the 1-hydroxypyrene accumulated in the tissues of exposed worms
422 can be regarded as a suitable indicator for assessing polychaete total PAH exposure: (i) the
423 relationship between 1-hydroxypyrene and several parent PAHs accumulated in animal tissues,
424 together with the corresponding total PAH sediment exposure levels and (ii) the relationship
425 between 1-hydroxypyrene levels and total PAH levels in the tissues of animals exposed to
426 different PAHs.

427 Accumulation of total PAH (TPAH) in relation to the sediment exposure concentration is
428 illustrated in Fig. 5. The TPAH tissue content was analyzed by both HPLC/F and GC/MS. There
429 is a strong positive concentration - response relationship in this experiment. Animals exposed to
430 five PAHs showed increasing accumulation of all five compounds with increasing exposure
431 concentration (Fig. 5). Each of the PAH compounds showed different rates of accumulation in
432 animal tissues, however, the total concentration of all 5 PAHs followed the increasing exposure
433 concentration.

434
435 Fig. 5.
436

437 As mentioned in the Materials and Methods section, worm tissues were extracted in two
438 steps: first in methanol, in order to extract soluble metabolites and, secondly, in dichloromethane
439 (DCM) in order to collect the remaining parent PAHs left in the tissues after the first extraction
440 step. It was not possible to analyze samples diluted in DCM using HPLC/F running
441 methanol/water gradient. Therefore, samples from the second extraction, diluted in DCM were
442 directly analyzed using GC/MS without further treatment, in order to keep most of the extracted
443 PAHs, as the amounts were predicted to be very low. There was a strong positive and significant
444 correlation between TPAH measured by GC/MS and HPLC/F (Spearman's Rank order correlation
445 $r_s = 1$, $P < 0.01$), indicating validity of both methods for this analysis (Fig. 6).

446
447 Fig. 6.
448

449 The sediment total PAH content after the experiment was analyzed using GC/MS,
450 recovery of the method ranged between 60-77% showing good recovery of the extraction method
451 for the sediment GC/MS analysis. The total PAH content of the sediment after the experiment was
452 lower than the nominal concentrations (Fig. 5), due to PAH loss during the experiment, such as
453 accumulation by animals and overlying water removal, and due to the spiking procedure.

454 A strong positive and significant correlation was observed between the sum concentration
455 of the five PAHs (TPAH) and 1-hydroxypyrene concentrations measured using HPLC/F and
456 GC/MS (for both methods: Spearman's rank-order correlation $r_s = 0.950$; $P < 0.01$.) (Fig. 7).

457
458 Fig. 7.

459 Note, that 1-hydroxypyrene is produced only from pyrene and that TPAH is the sum of
460 equal amounts of phenanthrene, anthracene, pyrene, B[a]p and B[k]f. In figure 7, numbers for 1-
461 hydroxypyrene concentration are 3-4 times lower than those for TPAH, since 1-hydroxypyrene is
462 produced only from pyrene. A strong positive correlation between concentrations of produced 1-
463 hydroxypyrene and accumulated TPAH was observed. The increase in the concentration of 1-
464 hydroxypyrene according to increase in the accumulated amount of TPAH, indicates that 1-
465 hydroxypyrene has potential as a useful biomarker for assessment of exposure to several different
466 PAHs (Fig. 7). It is evident from these results that the presence of other PAHs than pyrene in
467 sediment does not restrain the possibility of using 1-hydroxypyrene as a biomarker of total PAH
468 exposure. The animal organism is likely to be more stressed being exposed to different
469 xenobiotics than to pyrene only. Nevertheless, as long as there is pyrene in the PAH mixture, and
470 pyrene biotransformation is activated in the organism, 1-hydroxypyrene can indicate the
471 accumulation of PAHs into the organism over all uptake routes. The result above is not surprising,
472 since all worms under study were exposed to the same mixture of PAHs, with the same
473 proportions of each PAH. In environmental mixtures, on the other hand, the proportion of pyrene
474 to other PAHs varies between different samples. Therefore, the source of PAH contamination,
475 with its particular proportions of pyrene compound, should be taken into consideration, when
476 applying this method to field samples. Nevertheless, since pyrene is a constituent of all
477 environmental PAH mixtures, present in relatively high amounts (Levin, 1995; Jongeneelen et al.,
478 1985), the analysis of its metabolites could provide a useful screening tool for PAH exposure.
479 Note that the outcome provided by the measurements of 1-hydroxypyrene supposedly is used only
480 for screening of PAH exposure, meaning, that it can characterize the approximate level of PAHs
481 in the sediment. For the 1-hydroxypyrene value to represent a more quantitative index of exposure
482 to PAHs in an environmental mixture, many more studies should be conducted and especially the
483 proportions of single PAHs in the mixture should be taken into account.

484 Previous studies have shown that 1-hydroxypyrene can be used as a relative measure for
485 the total uptake of PAHs in humans (Levin, 1995; Jongeneelen, 2001) and fish (Ariese et al.,
486 1993). In human research, correlation studies have been made in order to establish the relationship
487 between pyrene and 1-hydroxypyrene and total PAH levels. For example, Tsai et al. (2004)
488 performed a correlation analysis to examine the relationship between total PAH exposure levels
489 and pyrene exposure levels in occupationally exposed workers, and the good correlation
490 established suggested, that the individual compound pyrene could be regarded as a suitable
491 indicator for total PAH exposure (Tsai et al., 2004). Another correlation analysis conducted by
492 Tsai et al. (2004) in order to examine the relationship between workers' urinary 1-hydroxypyrene
493 concentration and their corresponding total PAH exposure levels, indicated that the increase in
494 urinary 1-hydroxypyrene concentrations during the workweek could be fairly well explained by
495 PAH exposures arising from vehicle exhaust. Results presented in our study indicate that
496 relationships between 1-hydroxypyrene and total PAH exposure levels similar to those achieved in
497 human epidemiology can be established in benthic marine invertebrates. Thus, 1-hydroxypyrene
498 can be used as a relative measure of the total uptake of PAHs using exposed marine polychaetes

499 as well as fish bile from exposed fish. The value of the proposed simple method is, that it
500 integrates the bioavailability of sediment associated PAHs into the biomarker of PAH exposure. It
501 is well known, that similar sediment concentrations of PAHs can lead to very different internal
502 dose depending on the composition of the sediment, especially organic content and soot. To
503 estimate internal dose from total sediment concentrations without having a biological indicator
504 requires very extensive physicochemical measurements and calculations. The method proposed in
505 this study is not to be considered as a contribution to understanding of the exact fate of individual
506 compounds in a mixture of PAHs, but as method that takes advantage of the simple metabolic
507 pattern of pyrene, in order to crudely estimate general PAH exposure in the marine sediments.

508 To test the feasibility of quantifying 1-hydroxypyrene from worms exposed to different
509 PAHs, tissue concentrations of 1-hydroxypyrene from the five PAHs exposure experiment were
510 also quantified using HPLC/F and SFS. There was a strong positive and significant correlation
511 (Spearman's rank-order correlation, $r_s = 0.917$; $P < 0.01$) between 1-hydroxypyrene concentrations
512 measured by the two methods (Fig. 8).

513
514 Fig. 8.

515
516 There could be several explanations why data points representing data derived from worms
517 exposed to the highest PAH concentration (50 mg kg^{-1}) deviate more from the 1:1 ratio line than
518 data points from lower exposure concentrations. For instance, at the higher concentrations the
519 competition of different substrates for an enzyme may occur, causing higher variability of the
520 pyrene metabolites in the animal tissue. Hence, the interferences from those metabolites could
521 contribute to the SFS measurements, while the same metabolites would not interfere with 1-
522 hydroxypyrene during chromatographic separation on HPLC/F.

523 Nevertheless, the most important result achieved by these correlations is that the common
524 tendency is validated statistically. There is always a strong and significant positive correlation
525 between 1-hydroxypyrene and TPAH in the tissues and between 1-hydroxypyrene concentrations
526 measured by SFS and HPLC/F (Table 1).

527
528 Table 1.

529
530 Synchronous fluorescence spectroscopy of whole worm homogenate is a straightforward
531 method for detection of pyrene metabolites in marine deposit-feeding polychaetes, and the
532 complete analysis of one sample takes only few minutes, and requires much less instrumentation
533 than HPLC/F analysis. Giessing et al. (2003b) concluded that HPLC UV/F surpasses SFS as a
534 quantitative analytical technique. Nevertheless, when samples are treated with de-conjugating
535 enzymes, and large part of the protein-lipid matrix is precipitated, SFS can be applied for reliable
536 quantitative analysis of 1-hydroxypyrene, as it has been shown in the present study. During SFS
537 analysis conjugates that were not affected by enzyme treatment can contribute to fluorescence of
538 1-hydroxypyrene, therefore this technique might not offer a precise quantification of this
539 metabolite. HPLC/F analysis offer more precise measures of the 1-hydroxypyrene amounts in the
540 worm tissues than does SFS, since it separates conjugated metabolites from 1-hydroxypyrene.
541 However, SFS can indeed provide a useful and rapid screening tool for assessment of PAH
542 exposure in marine invertebrates.

543 Contrary to fish bile samples, the SFS method for polychaetes requires homogenization of
544 the tissue and enzymatic hydrolysis, making this method more labor intensive and expensive.
545 However, *Nereis diversicolor* is sessile and therefore a good indicator organism for local
546 pollution. In moderately polluted sediments, despite the fact that the fluorescence signal was
547 already below the detection limit at the spiking level of $1 \text{ mg pyrene kg}^{-1}$ sediment (dw) as
548 illustrated in Fig. 4, the SFS method may still be applied by pooling several individual worms
549 sampled at the same place in order to increase the total metabolite concentration.

550 Several studies have indicated that the sensitivity of the SFS method is sufficient for field
551 monitoring of PAH exposure using fish bile metabolites (Ariese et al., 1993; Lin et al., 1994). The
552 present study has shown that SFS analysis of only one metabolite - 1-hydroxypyrene - in
553 polychaete worm homogenate is sufficiently sensitive for assessment of total PAH exposure.
554 Presence of other PAHs in the contaminated sediment did not compromise the sensitivity of the
555 method, and the results achieved by SFS even at the highest exposure concentration correlated
556 well with the more precise HPLC/F method. Future experiments will reveal if similar correlations
557 between total PAH and 1-hydroxypyrene can be observed in benthic polychaetes from field sites.
558 However, the feasibility of such method, as measuring 1-hydroxypyrene by SFS in order to screen
559 total PAH exposure, will allow allocation of resources to other areas of risk assessment of
560 sediment, than costly preliminary chemical analysis of the environmental media and animal
561 tissues.

562 Taking into account the ongoing debate on the usefulness of biomarkers in environmental
563 risk assessment (ERA), where some authors emphasize advantages of biomarker application in
564 ERA (Martin-Diaz et al., 2004), others are criticizing the biomarker approach, suggesting to focus
565 our efforts on other methods (Forbes et al., 2006), and others while accepting criticism, suggest
566 further development of biomarkers applications in ERA (Van Gestel and Van Brummelen, 1996),
567 our study exhibits usefulness and applicability of the simple exposure biomarker 1-
568 hydroxypyrene. Biomarkers are often criticized for not being useful predictors of ecological effect
569 and for difficulties in linking responses from the individual to population levels (Forbes et al.,
570 2006). It is important to note that the present study proposes 1-hydroxypyrene as biomarker of
571 *exposure* and not *effect*. Therefore, it is not claimed that using 1-hydroxypyrene as a biomarker
572 compound would allow prediction of any adverse effect, and also especially on the higher level of
573 biological organization (e.g. population, ecosystem). Future experiments will show whether or not
574 different proportions of the compound pyrene in different environmental mixtures of PAHs
575 influence the usefulness of using 1-hydroxypyrene as surrogate for assessment of total PAH
576 levels. However, the method presented here, if applicable to field samples, could serve as an
577 effective, non-laborious and relatively low-cost screening tool for preliminary stages of sediment
578 ERA.

579
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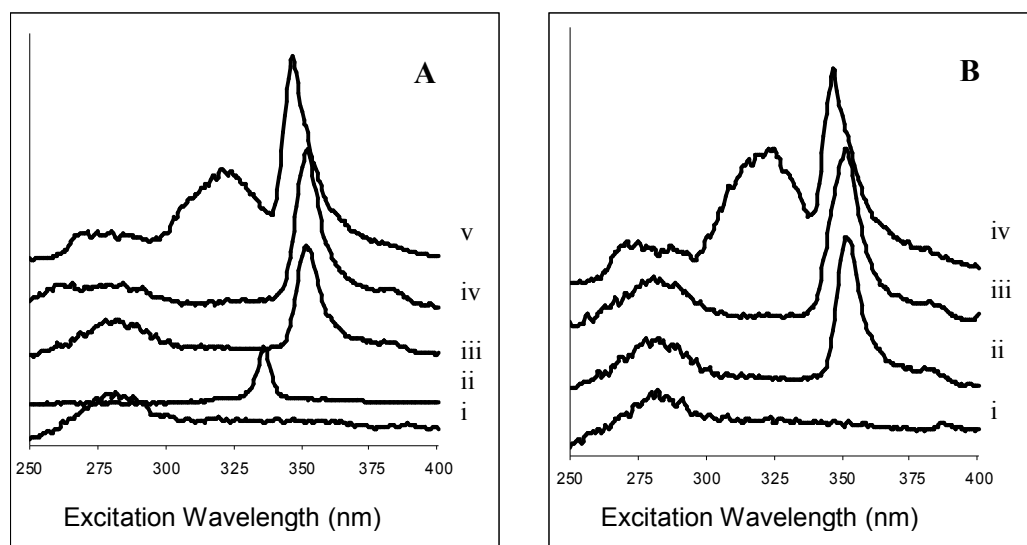


Fig. 1.

Synchronous fluorescence spectra of: A) tissue extracts of pyrene exposed worms. Unexposed worm (i); pyrene reference standard $1.4 \mu\text{g ml}^{-1}$ (ii); 1-hydroxypyrene reference standard, 60 ng ml^{-1} (iii), and tissue extracts, after (iv) and before (v) enzymatic treatment, spiking level of the sediment - 30 mg kg^{-1} ; B) extracts from worms exposed to five PAHs - before (iv) and after (iii) enzymatic treatment, spiking level of the sediment - 50 mg kg^{-1} , showing conjugated metabolites and the 1-hydroxypyrene peak, respectively; unexposed worm tissue sample (i) and 1-hydroxypyrene reference standard 60 ng ml^{-1} (ii).

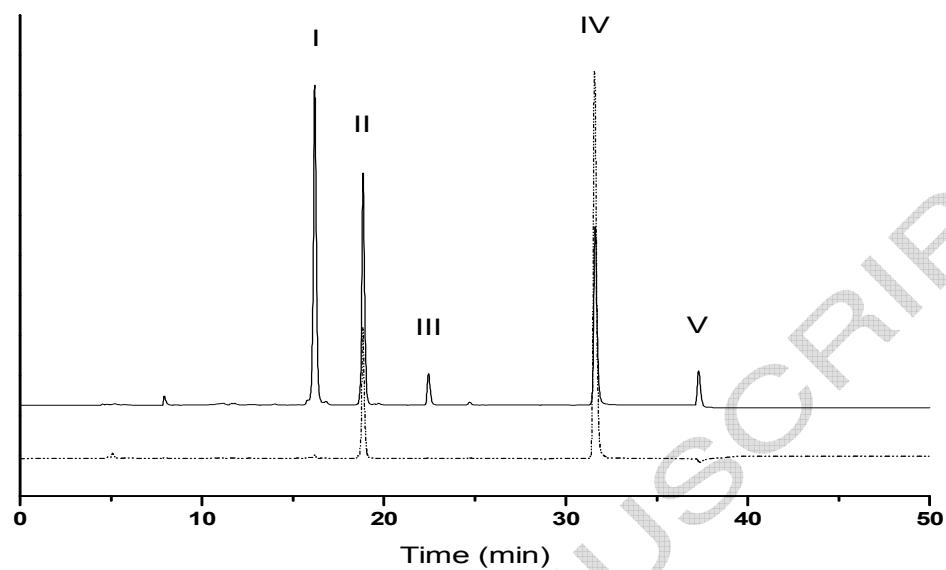


Fig.2.

Fluorescence chromatograms of tissue samples of worms exposed to pyrene, spiking level of the sediment - $10 \text{ mg kg}^{-1}(\text{dw})$.

Tissue sample prior treatment with β -glucuronidase-arylsulfatase (full line) revealing pyrene-1-glucuronide (I), pyrene-1-sulfate (II), pyrene-1-glucoside (III), 1-hydroxypyrene (IV) and pyrene (V). Tissue sample treated with enzyme (dashed line) revealing only two peaks - decreased peak of pyrene-1-sulfate and increased 1-hydroxypyrene.

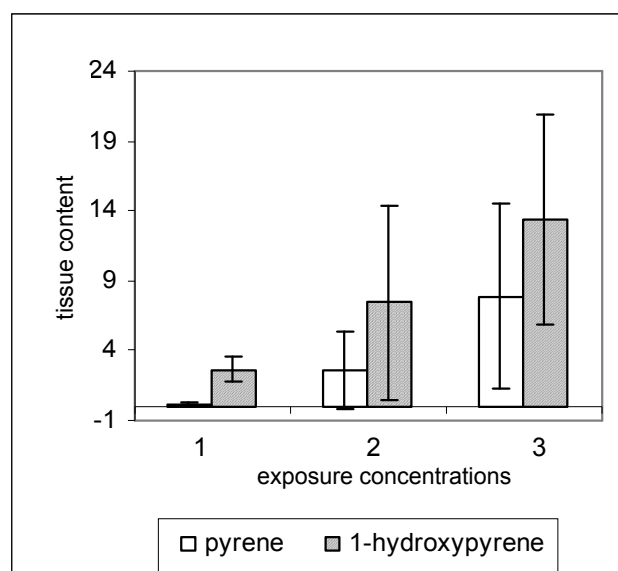


Fig.3.

Exposure to pyrene alone. Concentration - response relationship. Tissue extracts used had not been enzymatically hydrolysed. Accumulation of pyrene and production of 1-hydroxypyrene in animal tissues (ug g⁻¹ ww), measured by HPLC/F, follow the sediment exposure concentrations (mg kg⁻¹ dw).

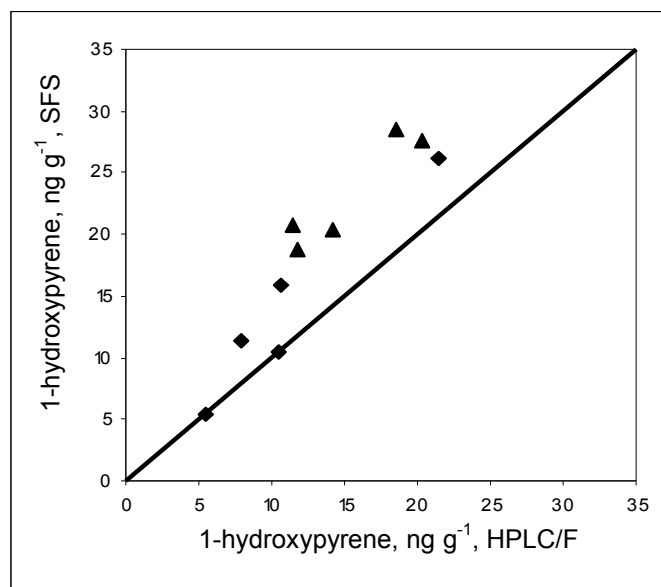


Fig.4.

Correlation of 1-hydroxypyrene concentrations measured by HPLC/F and SFS, in enzymatically hydrolyzed tissue samples (ng g^{-1} ww) from worms exposed to pyrene (\blacklozenge - 10 mg kg^{-1} dw; \blacktriangle - 30 mg kg^{-1} dw). The straight line indicates 1:1 ratio.

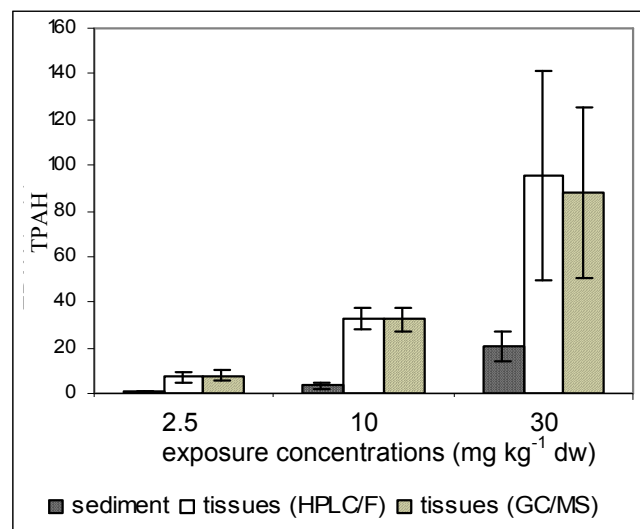


Fig.5.

Total PAH content (TPAH) in sediment ($\mu\text{g g}^{-1}$ ww) and animal tissues ($\mu\text{g g}^{-1}$ ww), from the five PAH exposure experiment. PAH content in tissues was measured by both HPLC/F and GC/MS; in sediment by GC/MS.

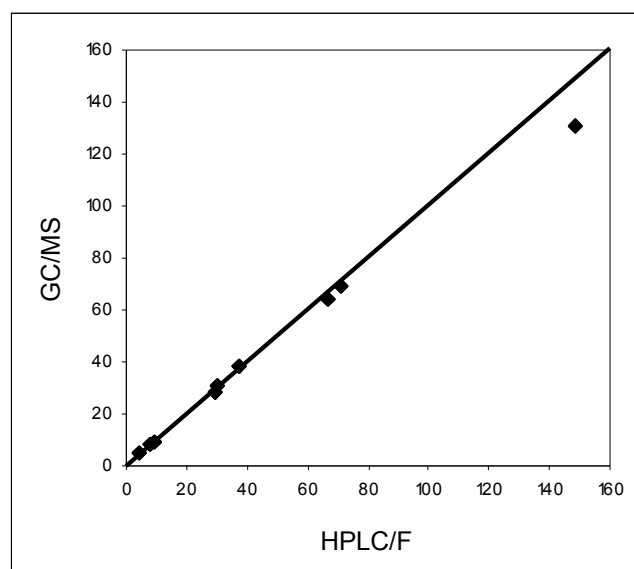


Fig. 6.
Correlation of TPAH values in tissues ($\mu\text{g g}^{-1}$ ww), measured by two analytical methods HPLC/F and GC/MS. The straight line indicates 1:1 ratio.

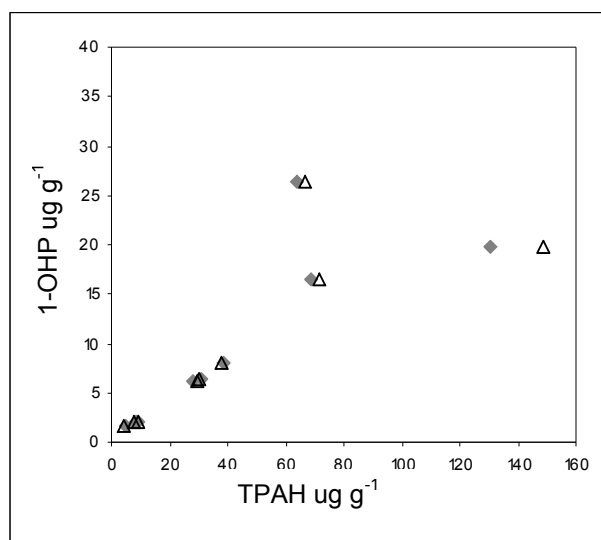


Fig. 7.

Correlation between 1-hydroxypyrene (1-OHP) concentrations and total PAH (TPAH) concentrations measured by HPLC/F (open symbols) and GC/MS (filled symbols) in the tissues of the exposed worms.

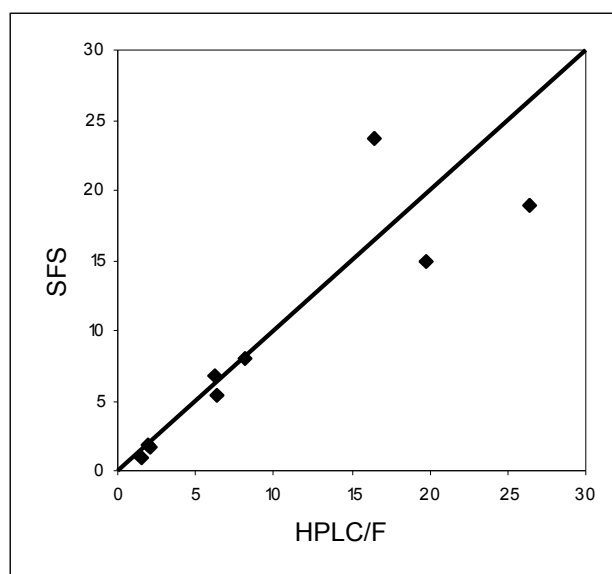


Fig. 8.
Correlation between 1-hydroxypyrene (1-OHP) concentrations ($\mu\text{g g}^{-1}$ tissue) measured by SFS and HPLC/F. The straight line indicates a 1:1 ratio.

Tables

Correlation	r_s value	P value
1-OHP (HPLC/F) vs. TPAH (HPLC/F)	0.950	$P < 0.01$
1-OHP (GC/MS) vs. TPAH (GC/MS)	0.950	$P < 0.01$
HPLC/F vs. SFS (1-OHP)	0.917	$P < 0.01$
HPLC/F vs. GC/MS (TPAH)	1	$P < 0.01$

Table 1. Results of correlation analysis of 1-hydroxypyrene (1-OHP) and Total PAH (TPAH) content of animal tissues, measured by three analytical methods (HPLC/F, SFS and GC/MS). r_s and P values identified by Spearman's rank-order correlation ($n=9$).