Chemical Derivatization and Shpol'skii Spectrofluorometric Determination of Benzo[a]pyrene Metabolites in Fish Bile

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The practical applicability and analytical performance of laser-excited Shpol'skii spectroscopy (LESS) for the determination of benzo[*a*]pyrene (B*a*P) metabolites was studied. Quantitation of these compounds in fish bile can provide insight into the B*a*P uptake from a contaminated aquatic environment (biomonitoring). Chemical derivatization of hydroxylated B*a*P metabolites with methyl iodide (to improve the compatibility with the Shpol'skii matrix) was tested. The methylation of phenolic metabolites was rapid and quantitative, but with B*a*P-dihydro diol metabolites, mixtures of methylation and elimination products were formed. An analytical procedure was developed for the quantitative determination of 3-OH-B*a*P, the major B*a*P metabolite detected in bile from flounder (*Platichthys flesus*). With LESS, the detection limit was 0.005 ng/mL (200 amol); the repeatability was 16%. The method was applied to a mesocosm experiment in which flounders were exposed to different sediments. The average B*a*P uptake from Rotterdam harbor sediment was 40 times higher than the uptake from Wadden Sea sand. Direct contact with the sediments was a major route of exposure. The usefulness of the method for the biomonitoring of polycyclic aromatic hydrocarbon stress in the aquatic environment is discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are considered an important class of environmental pollutants. PAHs may originate from incomplete combustion or petrochemical contamination and are relatively persistent in the environment. Many PAHs show mutagenic and/or carcinogenic activity[1] and may constitute a threat to human health. PAH pollution can also affect animal life, especially in the aquatic environment.[2,3] High incidences of liver tumors in fish at particular sites were found to be correlated to high levels of PAHs in the sediments.[4,5]

In order to monitor exposure to xenobiotics in the environment, the determination of their concentrations in the various environmental compartments may not be sufficient, since bioavailability is not taken into account. Biological monitoring, that is, the determination of a particular compound (or its metabolites) in a specific organism or tissue, can provide valuable information on the actual uptake rate.[6] Fish usually do not show considerable accumulation of PAHs.[7] Upon absorption, PAHs are rapidly metabolized into more polar derivatives that are stored in the gallbladder to be excreted.[8,9] Attempts to biomonitor PAH uptake by fish should therefore concentrate on PAH metabolites in excreta rather than on parent PAHs in tissue. Krahn and co-workers developed HPLC/fluorescence and GC–MS methods to determine PAH metabolites in fish bile.[10]

The potent carcinogen benzo[a]pyrene (B*a*P) is often used as a model compound to study the toxic effects of PAHs.[11] B*a*P is metabolized by hepatic enzyme systems into a number of mono- and polyhydroxylated derivatives. Some reactive species may form adducts with proteins or DNA, but most metabolites are rapidly excreted in the form of glucuronide, sulfate, or glutathion conjugates.[12] In laboratory experiments, the biotransformation products of B*a*P are usually analyzed by means of HPLC; to avoid problems with detection sensitivity, toxicologists can administer high doses of B*a*P or use radioactive material. In the field, the concentration of B*a*P metabolites in bile of feral fish could be used as an indicator of exposure to B*a*P and related PAHs in the area, but the detection of B*a*P metabolites requires extremely sensitive and selective methods. Bile of fish exposed to many different PAHs will contain an even more complex mixture of PAH metabolites that may interfere with the analysis. Using HPLC/fluorescence, Krahn et al. succeeded in detecting 3-hydroxy-B*a*P in some bile samples from a highly polluted site near Seattle,[13] but sub-ppb sensitivity would be needed for detection of BaP exposure in other, less polluted areas. As an extra complication, the bile volumes available are usually not sufficient for trace enrichment.

Shpol'skii spectrometry may offer the required sensitivity and selectivity. The method is based on the fact that some compounds, when cooled in a suitable crystalline matrix to cryogenic temperatures, will show highly resolved fluorescence spectra.[14,15] The technique combines fingerprint identification with excellent sensitivity and allows for the determination

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of complex mixtures without chromatographic separation.[16,17] The analytical performance can be further improved when a tunable laser is used as excitation source (laser-excited Shpol'skii spectrometry, LESS).[18,19] Shpol'skii spectra have been published for a large number of compounds, mainly nonpolar, planar, rigid molecules like PAHs that can substitute one or more solvent molecules in an *n*-alkane crystal.[15] In a few cases, the Shpol'skii effect was observed for more polar compound, like PAH metabolites.[20,21] Weeks and coworkers described a procedure to transform monohydroxybenz[*a*]anthracenes into less polar methoxy derivatives, which could subsequently be analyzed by means of LESS.[22] Recently, the same research group reported the derivatization and Shpol'skii spectra of a wide range of B*a*P metabolites: monohydroxy-B*a*P derivatives, B*a*P-dihydro diols, B*a*P-dihydro diol epoxide, as well as B*a*P-tetrahydro tetrol.[23]

In this paper we investigate the practical applicability of the Shpol'skii technique to the analysis of BaP metabolites in fish bile. The derivatization reaction was critically studied for phenolic and dihydro diol metabolites of B*a*P. An analytical method was developed for the quantitation of 3-OH-B*a*P in bile samples. The model fish studied was the flatfish species flounder (*Platichthys flesus*). Exposure to B*a*P was realized via parenteral injection or in mesocosm systems containing different sediments. Some field samples were also analyzed.

EXPERIMENTAL SECTION

Chemicals and Safety. All 12 monohydroxy-B*a*P isomers, B*a*P-*trans*-9,10-dihydro diol, B*a*P-*trans*-7,8-dihdyro diol, B*a*P-*cis*-4,5-dihydro diol, and B*a*P-*trans*-4,5-dihydro diol were obtained from the NCI Chemical Carcinogen Repositories (MRI, Kansas City, KS). Benzo[*a*]pyrene was purchased from Radiant Dyes (Wermelskirchen, Germany). Perylene-d_{12} was purchased from Merck Sharp & Dohme (Montreal, Canada). β-Glucuronidase (30 units/mL) with aryl sulfatase activity 20 units/mL was supplied by Merck. *n*-Octane was obtained from Janssen Chimica; all other solvents were Baker analyzed grade. The chemicals were used without further purification. Since B*a*P is a potent carcinogen, protective gloves should be worn when handling B*a*P solutions or during dissection of B*a*P-injected fish. Most B*a*P metabolites are considered nontoxic, but care should be taken when handling 2-hydroxy-B*a*P, 11-hydroxy-B*a*P, or B*a*P-*trans*-7,8-dihydro diol.[1] As the derivatization reaction is carried out with strong base and with the very toxic methyl iodide, the use of a fume cupboard and of protective clothing is strongly recommended.

Fish Exposure Studies. We studied flounder bile samples representing a wide range of B*a*P exposure levels. Highest exposure levels were realized by administering a single dose of B*a*P (parenteral injection in acetone/Mulgofen 620; 0.78 or 4.04 mg/kg of body weight). The fish were fed shrimp (*Crangon crangon*) until 2 days before injection and were sacrificed 48 h after injection. To simulate semichronic exposure to realistic B*a*P pollution levels, flounders were kept for 4 weeks in three different mesocosms: (1) moderately polluted Rotterdam harbor sediment (dredging class II, direct contact with the sediment was possible); (2) indirect exposure to Rotterdam harbor sediment (Wadden Sea sand bottom; food and water equilibrated with the polluted harbor sediment; (3) Wadden Sea sand bottom (control group). The PAH contents of the sediments (fine fraction only) were determined after wet sieving over a 63-μm Nylon filter, by means of HPLC with fluorescence detection.[24] The harbor sediment contained 450 ppb B*a*P and 800 ppb pyrene; the Wadden Sea sand contained 140 ppb B*a*P and 180 ppb pyrene (ng/g dry weight of fine fraction). The fish were fed until 2 days before sectioning to allow the accumulation of metabolites in the gallbladder and to reduce the confounding effects from different feeding habits. Finally, field samples were analyzed from flounders captured at the Wadden Sea and at a more remote part of the North Sea (53° 44′ N; 6° 30′ E). Bile was collected from the gallbladder by means of a syringe and stored in vials in the dark at −20 °C until further use.

Sample Treatment. Bile (20 μL) was diluted with water to 1 mL and incubated for 2 h at 37 °C with 20 μL of β-glucuronidase/aryl sulfatase solution to hydrolyze conjugated metabolites. Typically, the maximum yield was reached within 20–30 min. The free metabolites were quantitatively extracted by repeated extractions with *n*-hexane (4 times 3 mL). We observed that for the extraction of deconjugated PAH metabolites from bile samples, the use of solid-phase C_{18} cartridges often resulted in lower extraction efficiencies, less effective cleanup, and irreproducible yields. The latter was presumably caused by the varying concentrations of surfactants in the samples. For direct analysis of underivatized metabolites, hexane was evaporated in a stream of nitrogen and the residue dissolved in 2 mL of *n*-octane. In most cases, however, the volume of the extract was reduced to ca. 0.5 mL and the metabolites were derivatized according to a procedure adopted from Weeks et al.:[22] 2 mg of sodium hydride was washed three times with *n*-pentane in a flask under nitrogen atmosphere. Then 1 mL of dimethyl sulfoxide (DMSO) was added, and the mixture was stirred at 70 °C for several minutes until the formation of H_2 bubbles had ceased. Subsequently, the bile extract was added; the reaction mixture was vigorously stirred for another 10 min at 60 °C and then cooled to room temperature. Finally, 100 μL of methyl iodide was added; after several minutes of stirring the reaction was quenched with 4 mL of water. The methylated products were quantitatively extracted with 2 times 3 mL of *n*-hexane. For Shpol'skii analysis, the extract was concentrated and the solvent gradually replaced with *n*-octane in a gentle stream of nitrogen. The exact end volume was determined by weighing (ca. 400 μL). *n*-Hexane was preferred as extraction solvent over *n*-octane because of its lower boiling point and higher purity. For quantitation, perdeuterated perylene was added to the final analytical sample as an internal standard; 2×10^{-8} M for bile samples from the most polluted mesocosm, 2×10^{-9} M for the other samples. When a number of derivatizations had to be carried out, a slightly different procedure was followed: a larger volume of methyl iodide solution in basic DMSO was prepared and divided over the bile extracts in disposable glass vials at room temperature. This time-saving method also eliminates the risk of memory effects. For the methylation of phenolic B*a*P derivatives, both methods yielded equal results (cf. below).

Shpol'skii Spectrometry. For selective laser excitation a Quantel frequency-doubled Nd:YAG laser was used to pump an oxazine 170 dye laser; frequency mixing with the fundamental 1064-nm output yielded a tunable range from 409 to 419 nm. The beam intensity was typically 1 mW/3 mm^2 cross section; repetition rate 10 Hz. In case short-wavelength, nonselective excitation was needed, the dye laser output was frequency doubled to yield 348-nm radiation. In some cases, broad-banded xenon lamp excitation at 300 nm was employed. Four samples (ca. 10 μL) could be cooled simultaneously to 23 K by a CTI Cryogenics (Waltham, MA) closed-cycle helium refrigerator. The spectral resolution did not improve significantly when the samples were cooled to 10 K. Front-face illumination was applied; fluorescence emission was collected at a 20° angle, dispersed by a Spex 1877

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triple monochromator (1200 grooves/mm grating; resolution 0.1 nm), and detected by an intensified diode-array detector from Princeton Instruments (Type IRY 1024 GRB). For gated detection the Princeton Instruments GF-100 pulser unit was used.

RESULTS AND DISCUSSION

Direct Shpol'skii Analysis of BaP Derivatives. First, we explored the practical applicability of the Shpol'skii technique to the direct analysis of BaP metabolites (without chemical derivatization). In the past, quasilinear spectra of some monohydroxy metabolites had been reported for 3-OH-BaP and 6-OH-Bap,[20] and for 9-OH-BaP,[21] but no mention was made of applications to real analytical problems.

As expected, using a gold-plated copper sample holder with sapphire windows designed for optimal thermal conductivity and instantaneous solidification of small (10-μL) sample volumes, the phenolic metabolites 1-OH-BaP, 3-OH-BaP, and 9-OH-BaP all yielded narrow-banded spectra in *n*-octane at 23 K. Nevertheless, there is strong evidence that these analytes are not fully compatible with the crystalline *n*-octane host: (1) Some broad-banded background fluorescence was always observed; the relative intensity would increase when applying a less optimal cooling regime. (2) Spectra of 9-OH-BaP at different concentrations showed different site distributions. (3) Despite the good fluorescence quantum yields at room temperature,[20] detection limits were unexpectedly high (at best 10^{-8} M for 3-OH-BaP, using Xe lamp excitation at 300 nm, more than 1 order of magnitude worse than for the parent compound BaP under similar circumstances). At high concentrations (10^{-5} M), no relative decrease of the 0–0 emission line due to reabsorption was observed. These findings suggest that, as the result of the polar phenolic group, most analyte molecules freeze out of the crystalline matrix during the cooling procedure and give rise to a broad background or form nonfluorescent aggregates.[25] Consequently, the actual concentration of isolated analyte molecules that produce quasilinear emission is considerably lower than the analytical concentration. Apart from the obvious loss of sensitivity, the fact that the shape and intensity of the Shpol'skii spectra depend critically on concentration, cooling rate, or the presence of polar impurities in the matrix, will make proper quantitation in real samples very difficult.[26]

1-OH-BaP and 3-OH-BaP (but not 9-OH-BaP) were detected in bile of flounder after injection with BaP,[27] but one should not forget that the average daily intake of BaP by fish in a polluted environment will be several orders of magnitude lower than the applied dose. For reasons of sensitivity (and also repeatability), we concluded that Shpol'skii spectrometry is not a suitable method for the direct determination of monohydroxy-BaP metabolites in field samples and that derivatization would be required.

Evidently, we did not obtain analytically useful narrow-banded spectra for the still more polar dihydro diol metabolites (BaP-*trans*-9,10-dihydro diol, BaP-*trans*-7,8-dihydro diol, BaP-*cis*-4,5-dihydro diol, and BaP-*trans*-4,5-dihydro diol). Attempts to use more polar solvents or mixed solvents as a Shpol'skii host for these metabolites were unsuccessful.

Derivatization of Phenolic Metabolites. In order to render the analytes more compatible with the *n*-octane matrix, the derivatization procedure described by Weeks et al.[22] was adopted. Testing the reaction for 1-OH-BaP, 3-OH-BaP, and 9-OH-BaP, we found that the methylation of phenolic BaP derivatives with methyl iodide in basic DMSO is indeed a very rapid reaction: At high analyte concentration, the formation and methylation of a BaP phenolate is visualized by instantaneous color changes. Since the derivatization reagents do not interfere with the analysis, they can be added at large excess. In that case, the kinetics of the reaction are of the pseudo-first-order type and the derivatization is equally fast at low analyte concentrations. Quenching the reaction after 10 s or after 10 min resulted in equal yields. Analyzing the reaction mixtures with thin-layer chromatography, HPLC, and Shpol'skii spectrometry, we never detected any unreacted starting material or side products. In contrast with the derivatization of dihydrodiol metabolites (cf. below), the methylation of phenols was found to be a practical, straightforward, and quantitative reaction, irrespective of reaction time or temperature (20 or 60 °C).

The 12 monohydroxy derivatives of BaP were methylated and their Shpol'skii spectra recorded using nonselective laser excitation at 348 nm. In an *n*-octane matrix, the 12 methoxy derivatives all produced different fingerprint spectra, making isomer-specific determination possible (see Figure 1).

In the case of 3-OH-BaP, the improved host–guest compatibility after derivatization resulted in a 20-fold increase in quasilinear fluorescence intensity; the detection limit improved to 5×10^{-10} M (lamp excitation at 300 nm). Furthermore, the spectra were fully reproducible, irrespective of the cooling procedure, and concentration-dependent site distributions were not observed for any of the monomethoxy derivatives. In conclusion, the derivatization of phenolic metabolites prior to Shpol'skii analysis results in a considerable improvement of both sensitivity and reproducibility.

Derivatization of Dihydro Diol Metabolites. The bioactivation of BaP to mutagenic intermediates occurs primarily through monooxygenation at the 7,8 or at the 9,10 position. The resulting epoxides are usually hydrolyzed to the respective dihydro diols, which may either be metabolized further or conjugated and excreted.[28] The development of a sensitive, isomer-specific technique for the analysis of dihydrodiol metabolites in biological matrices would be of great importance. Weeks and colleagues presented LESS spectra of the derivatization products of BaP-*trans*-7,8-dihydro diol and BaP-*trans*-9,10-dihydro diol.[23] Apparently, the methylation of vicinal, aliphatic hydroxy groups would proceed in the same manner as in the case of phenolic compounds, and no mention was made of the formation of side products. Since, however, previous attempts in our laboratory to derivatize BaP dihydrodiols always led to more than one product, we decided to study the reaction in more detail. When the reaction was carried out at 60 °C, as outlined in the Experimental Section, the derivatization product of BaP-*trans*-9,10-dihydro diol showed room-temperature fluorescence excitation and emission spectra characteristic for monohydroxy-BaP derivatives, but very different from that of the starting material, indicating that the chromophore had undergone an important change. GC–MS analysis revealed the presence of two compounds with identical mass spectra: molecular ion 282; major fragments 267 ($M - CH_3$) and 239 ($M - CH_3OC$), analogous to the fragmentation of methoxy naphthalene.[29] Even when direct-inlet/chemical ionization mass spectrometry was applied to the product mixture, no compound with molecular ion m/z = 314 was detected. Finally, the reaction products were fractionated by means of preparative normal-phase HPLC (Lichrosorb Si-60 5-μm packing; mobile phase 100% *n*-hexane) and unambiguously identified as 9-methoxy-BaP and 10-methoxy-BaP. Their

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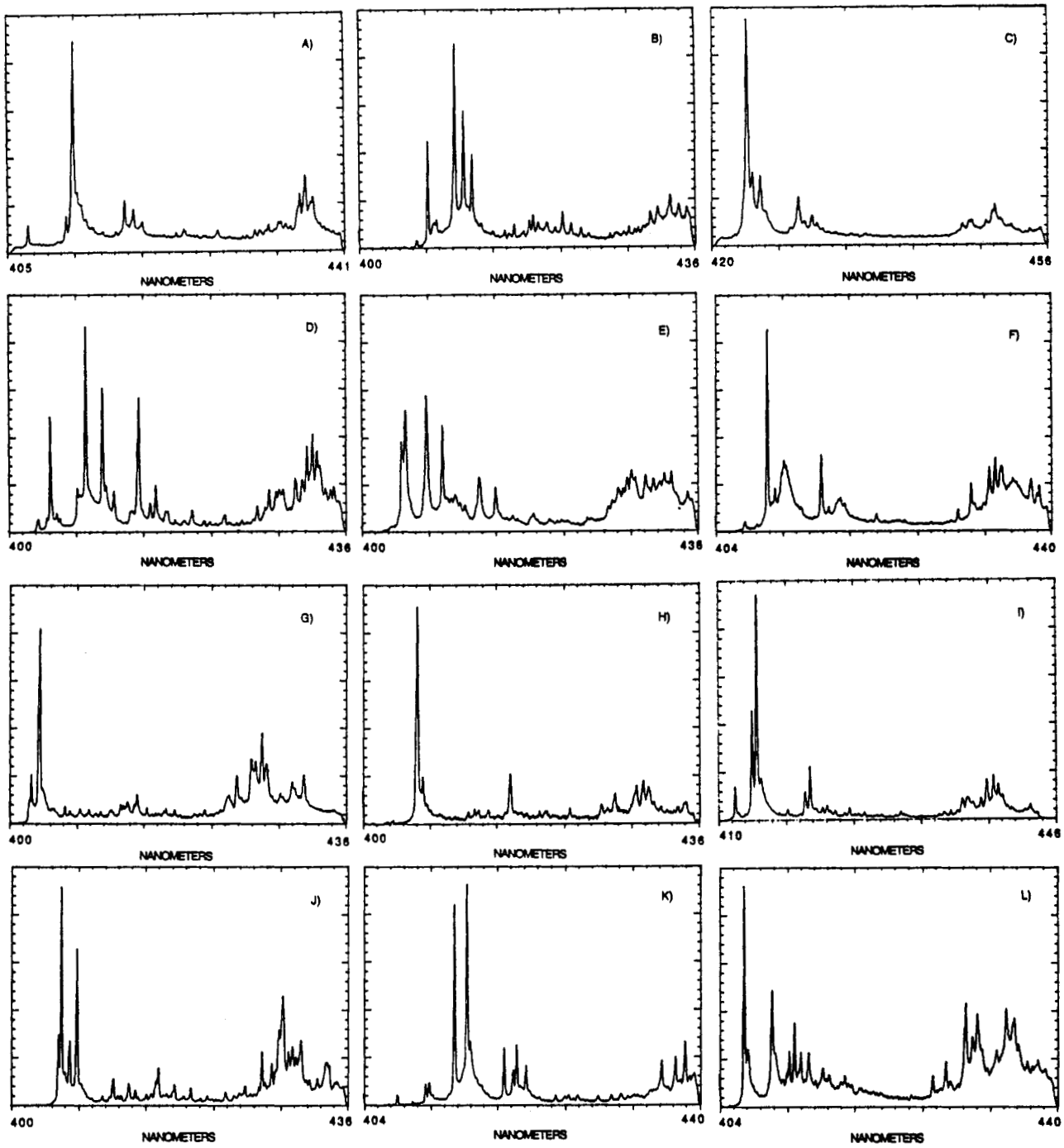

Figure 1. Shpol'skii spectra in *n*-octane of phenolic B*a*P metabolites after derivatization, using nonselective laser excitation at 348 nm: (A) 1-methoxy B*a*P, 10^{-7} M; (B) 2-methoxy-B*a*P, 10^{-6} M; (C) 3-methoxy-B*a*P, 2×10^{-7} M; (D) 4-methoxy-B*a*P, 10^{-6} M; (E) 5-methoxy-B*a*P, 10^{-6} M; (F) 6-methoxy-B*a*P, 10^{-6} M; (G) 7-methoxy-B*a*P, 10^{-6} M; (H) 8-methoxy-B*a*P, 10^{-6} M; (I) 9-methoxy-B*a*P, 10^{-6} M; (J) 10-methoxy-B*a*P, 10^{-6} M; (K) 11-methoxy-B*a*P, 10^{-6} M; (L) 12-methoxy-B*a*P, 10^{-6} M.

respective Shpol'skii spectra were identical to those presented in Figure 1I,J. Apparently, at elevated temperatures and in prolonged contact with a strong base, B*a*P-*trans*-9,10-dihydro diol undergoes elimination of water, yielding one of two possible phenolates which are subsequently methylated as soon as CH_3I is added to the reaction mixture. The driving force of the reaction is, of course, the restoration of the full aromaticity of B*a*P. In a similar way, the methylation of B*a*P-*trans*-7,8-dihydro diol yielded a mixture of 7-methoxy-B*a*P and 8-methoxy-B*a*P when carried out at elevated temperatures. Methylation of B*a*P-*trans*-4,5-dihydro diol led to a mixture of 4-methoxy-B*a*P, 5-methoxy-B*a*P, and a third, unidentified compound. The monomethoxy isomers were identified with help of the Shpol'skii spectra from Figure 1.

In an attempt to suppress the elimination reaction, the basic DMSO solution was first cooled to 20 °C, and the time lapse between the addition of B*a*P-*trans*-9,10-dihydro diol and methyl iodide was minimized. Best results were obtained when CH_3I was added prior to the metabolite. The room-temperature methylation of B*a*P-*trans*-9,10-dihydro diol yielded a main product with fluorescence excitation- and emission spectra similar to that of the starting reagent. Using thin-layer chromatography (RP-18 plates from Merck, mobile phase acetonitrile/water 90:10 v/v), a new product was detected, as well as at least one minor side product with the retention factor of 9-methoxy-B*a*P. We found no evidence of unreacted starting material. The direct-inlet mass spectrum of the product mixture showed the presence of a compound with $m/z = 314$, with major fragments 283 ($M - CH_3O$) and

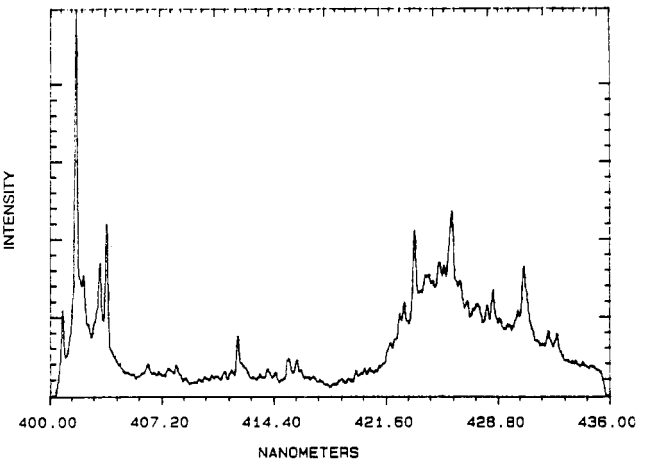

Figure 2. Shpol'skii spectrum in *n*-octane of purified main product after room-temperature derivatization of BaP-*trans*-9,10-dihydro diol. Concentration = 1×10^{-7} M; λ_{exc} = 348 nm.

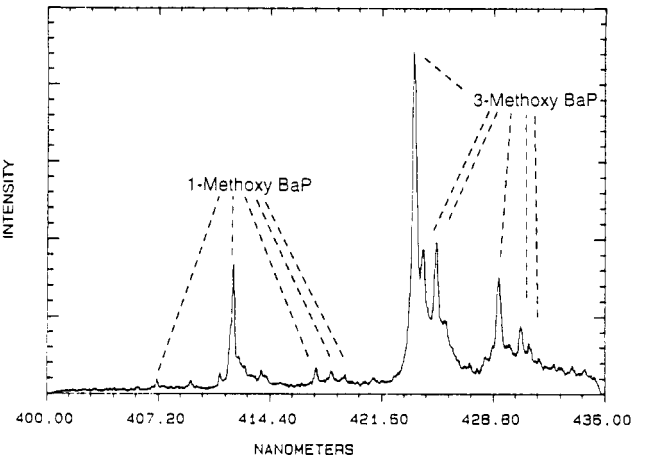

Figure 3. Shpol'skii spectrum of methylated flounder bile sample (0.78 mg/kg BaP injected), featuring 1-methoxy-BaP and 3-methoxy-BaP. λ_{exc} = 348 nm.

252 ($M-2CH_3O$), which is in full agreement with the expected dimethylated product. The spectrum also contained the masses 282, 267, and 239, characteristic for monomethoxy BaP (elimination products). Using preparative normal-phase HPLC (mobile phase: hexane/2-propanol 95:5 v/v), the main product was separated from the side products 9-methoxy-BaP and 10-methoxy-BaP and its Shpol'skii spectrum in *n*-octane solution could be recorded (Figure 2).

All evidence suggests that the major product of the room-temperature derivatization of BaP-*trans*-9,10-dihydro diol was indeed the desired BaP-*trans*-9,10-dihydro dimethoxy. The Shpol'skii spectrum shown in Figure 2, however, bears no resemblance to that presented by Weeks et al.,[23] which cannot be explained by differences in excitation wavelength or cooling rate but rather indicates that the derivatization reaction may also lead to other products than the three compounds identified in this study. Unfortunately, even at room temperature, the formation of elimination products could not be entirely avoided.

We conclude that great care has to be taken when using the derivatization reaction described above for the analysis of BaP-dihydro diols: the formation of the corresponding dihydro dimethoxy compound may not be quantitative, while, on the other hand, the detection of 4- and 5-methoxy-BaP, 7- and 8-methoxy-BaP, or 9- and 10-methoxy-BaP does not necessarily indicate the presence of the corresponding phenolic metabolites in the original sample. Similar problems were encountered by Jacob et al.:[30] upon treatment of pyrene 4,5-dihydro diol in rat urine with strong acid (prior to methylation with diazomethane and GC analysis), large amounts of 4-hydroxy-pyrene were formed. An extra complication for quantitative applications is the fact that the appearance of the Shpol'skii spectrum (site distribution and relative intensity of the broad-banded background) was found to be dependent on concentration and cooling rate. The same phenomenon has been observed for other, not fully compatible host–guest combinations, e.g. acenaphthene in *n*-hexane.[25]

Analysis of BaP Metabolites in Fish Bile. In a first explorative study, bile samples were analyzed from fish that had received a high dose of BaP via injection. Before hydrolysis, no fluorescent metabolites could be extracted with hexane, indicating a high degree of conjugation.[12] A typical Shpol'skii spectrum of a bile extract after enzymatic hydrolysis and methylation at 60 °C is shown in Figure 3. Nonselective laser excitation at 348 nm was employed, in order to be able to determine all metabolites simultaneously; the overall dilution factor was 1000. The spectrum is dominated by 3-methoxy-BaP and 1-methoxy-BaP (compare with reference spectra in Figure 1C,A); the relative contribution of the latter varied considerably between individuals: between 7 and 26% of the total amount of metabolites detected. In some samples, using longer detector exposure times, traces of 7- and 8-methoxy-BaP, or of 9- and 10-methoxy-BaP could be detected. These compounds were not detected (7-, 8-, or 10-methoxy-BaP) or were present at a much lower level (9-methoxy-BaP) when the same bile samples were derivatized at room temperature, indicating that they were elimination products from their respective diols. The amount of 3-methoxy-BaP and 1-methoxy-BaP determined did not depend on derivatization conditions. Dimethyl derivatives of BaP-dihydro diol metabolites were not observed directly, which can be explained by the fact that their detectability in Shpol'skii matrices is 1 or 2 orders of magnitude lower than that of monomethoxy derivatives, as was also noticed by Weeks and co-workers.[23]

The fact that 3-OH-BaP and 1-OH-BaP are major metabolites in fish bile is in full agreement with the measurements of Krahn et al.[13] Within 48 h following parenteral injection, generally more than 50% of the administered dose had accumulated in the gallbladder as (conjugated) 3-OH-BaP. At present, we have no explanation for the observation that BaP-*trans*-9,10-dihydro diol and BaP-*trans*-7,8-dihydro diol contribute only marginally (<1%) to the (hydrolyzable) BaP metabolite profile in flounder bile.

Because of the fact that (1) BaP is predominantly excreted in the bile of flounder as 3-OH-BaP, (2) the derivatization of 3-OH-BaP is a fast and quantitative reaction, and (3) 3-methoxy-BaP yields good and reproducible Shpol'skii spectra, it was decided to develop a procedure for the quantitative determination of trace levels of 3-OH-BaP in fish bile and to use the latter as a marker compound for the biomonitoring of BaP uptake from a polluted environment.

Quantitative Determination of 3-OH-BaP. In the field, fish are exposed to a complex mixture of xenobiotics, including PAHs. Since each compound may be biotransformed into several different metabolites, the composition of excreta (e.g. bile fluid) is likely to be even more complex. For the spectrofluorometric determination of a specific analyte in an extract containing a multitude of fluorescent compounds, it is generally advantageous to choose the excitation and emission wavelengths close to each other, and so reduce the number of potential spectral interferences. Furthermore, in a Shpol'skii matrix, only the S_1–S_0 part of the excitation

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spectrum consists of narrow lines[15] and is suited for selective excitation with a narrow-banded light source. The optimal excitation wavelength for 3-methoxy-BaP was found to be 418.36 nm, only 5.2 nm apart from the 0–0 transition, the most intense emission line. Stray light was very effectively rejected by a triple monochromator. the remaining sources of background noise in spectra of solvent blanks were detector thermal noise, detector read-out noise, Raman lines, and quasilinear emission from impurities (e.g. perylene). In some cases, time-resolved detection was applied to reduce thermal noise or to distinguish between Raman lines and quasilinear fluorescence. The main emission line of 3-methoxy-BaP, however, did not suffer from spectral overlap. In practice, gating was found to decrease the absolute sensitivity of our intensified diode array detector and turned out to be of limited value for the analysis of real samples. The limiting factor in bile extracts was usually broad background fluorescence, and the average fluorescence lifetime of the background was not significantly different from that of the analyte (ca. 10 ns).

For quantitation, an internal standard must be added to the analytical sample to compensate for variations in sample thickness, laser power, and optical alignment. In order to assure proper correction for laser output fluctuations (meanwhile saving time as well), the internal standard should be excitable at the wavelength chosen for the analyte and should have a sufficiently strong emission line in the emission window covered by the multichannel detector. Perdeuterated perylene was found to meet the above requirements; ratioing the peak areas of the 0–0 emission lines, a straight calibration curve was obtained for 3-methoxy-BaP in the concentration range of interest (3×10^{-11}–1×10^{-8} M). The perylene-d_{12} concentration was kept constant at 2×10^{-9} M. Photochemical decomposition of the analyte was observed but was less than 5% during the usual irradiation time of 120 s. To assure correct quantitation, the calibration solutions were irradiated during the same period.

The absolute detection limit (S/N = 3) for 3-methoxy-BaP, using laser excitation at 418.36 nm, was found to be 5×10^{-12} M (50 amol) in *n*-octane solutions. The detection in bile extracts was not seriously affected by matrix interferences: When the sample treatment was carried out without overall dilution (provided that sufficient bile was collected), the detection limit was still 2×10^{-11} M or 0.005 ng/mL. For most samples, we used an overall dilution factor of 20; in that case the detection limit was 2×10^{-10} M or 0.05 ng/mL in the original sample, which was sufficient to detect exposure to BaP in all samples from the mesocosm experiment. The repeatability of the method (four replicates of sample extraction and determination) was 16%. Every day, a standard solution was analyzed to check for any deviations from the calibration curve. Because of the selectivity of excitation, a small change in laser wavelength would lead to gross analytical errors, but that was never observed.

Biomonitoring of BaP Exposure. Laser-excited Shpol'skii spectrometry of 3-OH-BaP was applied to a mesocosm study in which flounders were exposed during 4 weeks to three degrees of pollution, as described in the Experimental Section. Mesocosms 1 and 3 reflect the range of PAH pollution levels encountered in the Dutch coastal waters and estuaries. Mesocosm 2 was designed to find out what route of exposure contributes most significantly to the total BaP uptake. Hydrolyzed bile samples were derivatized at room temperature. After addition of the internal standard, the extracts were cooled to 23 K and their Shpol'skii spectra recorded using selective laser excitation at 418.36 nm. For the mesocosm samples, short-wavelength excitation at 348-mm excitation could not be used because of two reasons: limited sensitivity and spectral overlap with emission bands

Table I. Pyrene and BaP Metabolite Concentrations in Fish Bile after Exposure to Different Mesocosms[a]

	3-OH-BaP (ng/mL)	1-hydroxypyrene (ng/mL)
(1) harbor sediment (direct contact possible)	50 ± 36 (n = 9)	15900 ± 6700 (n = 23)
(2) harbor sediment indirect (PAH uptake through food and/or water)	7.7 ± 2.4 (n = 4)	2600 ± 1500 (n = 16)
(3) sand bottom	1.2 ± 0.1 (n = 3)	800 ± 480 (n = 26)

[a] Data are expressed as the arithmetic mean ± standard deviation (number of samples).

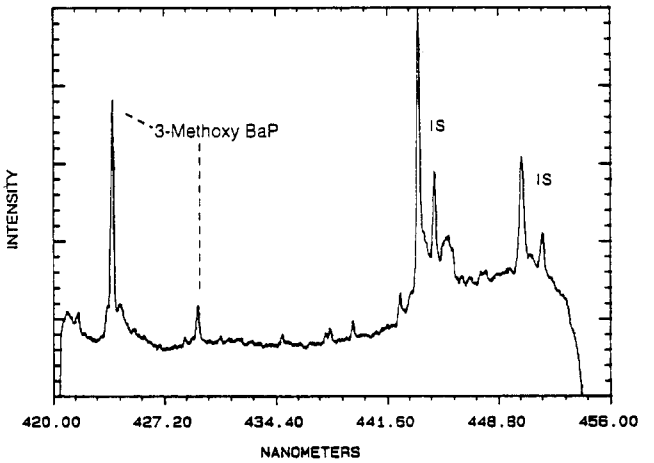

Figure 4. Shpol'skii spectrum of methylated bile extract from Wadden Sea mesocosm, featuring 3-methoxy-BaP. Site-selective laser excitation at 418.36 nm; IS = perylene-d_{12}.

from 1-methoxypyrene (1-hydroxypyrene is usually present at much higher levels in fish bile; see Table I). Notwithstanding the 20-fold dilution caused by the sample workup, 3-methoxy-BaP could be detected in all samples, even from mesocosm 3 (Figure 4). The multiplet structure from Figure 1C has disappeared as the result of site-selective excitation. The analytical results, summarized in Table I, show that fish exposed to Rotterdam harbor sediment had absorbed and metabolized 40 times more BaP than fish from the Wadden Sea sand basin. Furthermore, fish from the second mesocosm showed only a 6-fold increase, indicating that some uptake of BaP can take place through the water phase or through the diet[31] but that direct contact with the sediment is the major route of exposure for a bottom-dwelling fish like flounder. Direct absorption through skin or gills or ingestion of PAH-containing particles may both be important factors.

Table I also lists 1-hydroxypyrene levels determined by means of synchronous fluorescence spectrometry (SFS).[32] Although 1-methoxypyrene can be measured with Shpol'skii spectrometry in an *n*-octane matrix,[33] the relatively high concentrations allowed the determination (in a large number of samples) with a faster, more conventional method. The 1-hydroxypyrene data show a trend similar to the results for 3-OH-BaP, but the 1-hydroxypyrene concentrations are a factor of 300–600 higher, which is not explained by the relative contents of the parent PAHs in the sediments (see Experimental Section) nor by the fact that 3-OH-BaP is not the only metabolite of BaP. Apparently, the bioavailability of

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(32) Ariese, Freek; Kok, Steven J.; Verkaik, Marieke; Gooijer, Cees; Velthorst, Nel H.; Hofstraat, Johannes W. *Aquat. Toxicol.*, in press.

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pyrene is much higher than that of BaP, which agrees with the kinetic studies of Landrum.[6] The standard deviations reported in Table I are an indication of the usual biological spread.[8]

Finally, a limited number of field samples were analyzed: A pooled bile sample from flounders from the Wadden Sea was found to contain 1.6 ng/mL 3-OH-BaP, comparable to the levels encountered in the samples from mesocosm 3 (Wadden Sea sand bottom). Considerably lower exposure to BaP takes place at open sea: two bile samples from the North Sea contained levels of 3-OH-BaP just above the detection limit (ca. 0.005 ng/mL).

CONCLUSIONS

Direct Shpol'skii analysis of underivatized monohydroxy-BaP metabolites in fish bile is possible but has its drawbacks. The technique could be used in laboratory studies if unambiguous isomer-specific identification of biotransformation products is required. These analytes, however, are not fully compatible with the low-temperature *n*-octane host. Consequently, the spectra depend rather critically on experimental conditions, and the sensitivity is insufficient for most field applications. A rapid and quantitative derivatization reaction with methyl iodide in basic DMSO yields methoxy derivatives that give intense, high-quality Shpol'skii spectra in *n*-octane matrices and which are very suitable for trace determination with LESS.

The derivatization of BaP-dihydro diol metabolites was found to lead to a mixture of the dimethylated analyte and elimination products; the relative yields depend critically on reaction conditions. Even when alternative, straightforward reaction schemes will be found that yield quantitative results, the detectability of the nonplanar dihydro dimethoxy compounds in a Shpol'skii matrix is not nearly as good as that of the monomethoxy-BaP derivatives. The isomer specificity of the method could be useful for the study of BaP metabolism pathways, but the limited sensitivity is expected to hamper most applications.

BaP administered to flounders through parenteral injection was mainly excreted as conjugated 3-OH-BaP; 1-OH-BaP was the second major metabolite. Traces of 9-OH-BaP, BaP-*trans*-9,10-dihydro diol and BaP-*trans*-7,8-dihydro diol were identified in some samples.

An important finding is that, within 48 h following parenteral injection, generally more than 50% of the administered dose had accumulated in the gallbladder as (conjugated) 3-OH-BaP. This means that, even without induction, the mixed-function oxygenase (MFO) activity in flounder liver is rather high, and certainly not a rate-limiting factor if fish are exposed to submicrogram amounts of BaP in the field. Thus, the 3-OH-BaP concentration in bile is a straightforward indicator of the amount of BaP absorbed, not confounded by differences in metabolic activity. For the biomonitoring of BaP exposure in the field, it is an additional advantage that the metabolite concentration in bile reflects only the most recent uptake. It is, therefore, a good indicator of BaP stress in the area of capture.

An analytical procedure was developed for the quantitative determination of 3-OH-BaP in fish bile. By use of enzymatic hydrolysis, chemical derivatization, and laser excited Shpol'skii spectrometry, the detection limit is as low as 0.005 ng/mL, which is amply sufficient for the biomonitoring of BaP uptake in the Dutch coastal waters. Some extra effort will be required to monitor the much lower levels of BaP pollution at open sea. Application of the technique to a mesocosm experiment revealed that exposure to PAH-contaminated harbor sediment leads to a strongly increased uptake of BaP compared to the reference group. Direct contact with the sediment and/or ingestion of particles are major uptake routes.

The 1-hydroxypyrene levels in bile correlate well with the 3-OH-BaP data, but the absolute amounts of pyrene absorbed and metabolized are more than 2 orders of magnitude larger than that of BaP. On the other hand, the pyrene content of the sediments was not even twice as high as that of BaP: the PAH uptake profile is clearly very different from the PAH profile in the sediment. This illustrates the value of a biomonitoring approach: the actual integrated exposure of an organism, which is the resultant of environmental levels and the bioavailability of the compound via various uptake routes, is measured directly. When PAH metabolite determination in the bile of bottom-dwelling fish gains more widespread acceptance as a useful tool for the biomonitoring of PAH exposure in the aquatic environment, then fast and cheap analytical methods that can be applied to large numbers of samples will undoubtedly be preferred. In that case, determination of 1-hydroxypyrene with synchronous scanning fluorometry[32] or measurement of "total bile fluorescence" with HPLC[34] would be very useful as a simple screening method. That approach seems justified, since comparison of our data in Table I with the results of Krahn and co-workers[13] indicates that the PAH metabolite profile in bile of flatfish appears to be quite constant. For a limited number of samples, one could use a more sensitive and specific technique like laser-excited Shpol'skii spectrometry to quantitate the relatively small, but toxicologically relevant, uptake of potent carcinogens like BaP.

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