Environ. Sci. Technol. **1999**, *33,* 2710−2714

Hydroxylated PAHs in Bile of Deep-Sea Fish. Relationship with Xenobiotic Metabolizing Enzymes

E. ESCARTÍN AND C. PORTE*

Environmental Chemistry Department, IIQAB-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

Polycyclic aromatic hydrocarbon (PAH) pollution in deep-sea environments (1500−1800 m depth) has been assessed by measuring bile PAH metabolites in deep-sea fish. Five species from the NW Mediterranean were selected for the study: *Coryphaenoides guentheri*, *Lepidion lepidion*, *Mora moro*, *Bathypterois mediterraneus,* and *Alepocephalus rostratus*. Bile crude samples were directly analyzed by HPLC-fluorescence at the excitation/emission wavelengths of benzo[*a*]pyrene (380/430 nm). Differences among sampling sites were recorded, which suggests that coastal discharges of contaminants may reach these remote areas. Subsequently, a number of bile samples were hydrolyzed and analyzed by gas chromatography−mass spectrometry (GC-MS) for the determination of individual PAHs. 1-Pyrenol and 2-phenylphenol were among the most abundant compounds detected. The results obtained confirm the long-range transport of PAHs to deep-sea environments, subsequent exposure of fish inhabiting those remote areas, and its ability to metabolize and excrete them through the bile. The data also describe hepatic enzymes (cytochrome P450 and glutathione *S*-transferases) that appear to be as catalytically efficient as those in shallow water species.

Introduction

Deep-sea regions are poorly studied areas encompassing about 75% of the biosphere, in which abiotic and biotic factors show relatively little variation in both time and space. These regions are characterized by the absence of sunlight, depths greater than 1000 m, elevated pressures, and low temperatures.

However, despite their remoteness, those environments are reached by man-made pollutants. Several models suggested deep-sea sediments as the final accumulation site of organochlorine compounds (*1*). Thus fish living in association with sediments have merited considerable attention, and the bioaccumulation by deep-sea organisms of foreign organochlorine compounds originating from land-based activities has been documented (*2*−*5*). Research has also demonstrated that polycyclic aromatic hydrocarbons (PAHs) occur in remote deep-sea sediments (*6, 7*). However its bioaccumulation and/or metabolism by deep-sea organisms has been less studied (*8*).

PAHs exposure in aquatic organisms is often assessed by measuring the concentration of PAHs in their tissues. However, fish caught at highly polluted sites often showed only trace levels in the tissue due to its ability of metabolizing PAHs (*9*). Thus, alternative techniques have been developed in order to assess PAHs exposure in fish, viz. the determination of PAHs excreted through the bile as conjugated metabolites. Laboratory studies have demonstrated that the presence of PAH metabolites in bile is well correlated with levels of exposure (*10*−*13*), and this trend has been corroborated in a number of field studies (*14*−*16*).

Xenobiotics within the organism are subjected to a suite of reactions that will end up facilitating their excretion. In all eukaryotes, cytochrome P450 system and glutathione *S*-transferases play a key role in the biotransformation (monooxygenation and conjugation) of lipophilic foreign chemicals such as PAHs. Substantial differences in monooxygenase or transferase activities and number of isoenzymes are reported for marine organisms depending on habitat, pollutant load, etc. (*17, 18*). Deep-sea fish have evolutionary adaptations to extreme environmental conditions. Metabolic rates of deep-sea fish decrease with depth as a result of several interacting factors, viz. low temperatures, physiological acclimation to low food levels, and poor locomotory capabilities (*19*). Hence, strong differences respect coastal species in terms of xenobiotic metabolizing enzymes could be anticipated (*20*). However, very little work has focused upon the potential enzymatic systems that may be involved in xenobiotic biotransformation in deep-sea organisms (*21*).

This paper selected five fish species—*Coryphaenoides guentheri, Lepidion lepidion, Mora moro, Bathypterois mediterraneus,* and *Alepocephalus rostratus*—from the NW Mediterranean (1500−1800 m depth) to better understand the degree of exposure of deep-sea fish to PAHs. The Western Mediterranean receives urban and industrial wastewater discharges from bordering countries. Monitoring surveys have been carried out along the coast using fish as biomonitors (*22, 23*); however, nothing is known about the contamination of deep-sea biota in this area. Thus, the purpose of this study was (a) to assess the level of pollution of Western Mediterranean deep-sea regions by using different feral fish species as sentinel organisms and (b) to investigate the relationship, if any, between bile PAHs metabolites and xenobiotic metabolizing enzymes in these fish.

Materials and Methods

Sample Collection and Storage. Deep-sea fish—*Coryphaenoides guentheri, Lepidion lepidion, Mora moro, Bathypterois mediterraneus,* and *Alepocephalus rostratus*—were collected by trawling from three stations located in the Western Mediterranean at a depth ranging from 1500 to 1800 m (Figure 1). Once on board, the liver was immediately dissected, frozen in liquid nitrogen, and stored at −80 °C. The gall bladder was also dissected and stored in dark glass vials at −20 °C.

Fluorescent Aromatic Compounds (FACs) in Bile. Bile crude samples were analyzed by HPLC with fluorescence detection according to Krahn et al. (*24*). The analytical column was a 15 × 0.46 cm HCODS, C18, 5 μM (Perkin-Elmer), fitted with 10 × 4 mm guard cartridges of Hypersil PAH (Shandon HPLC). The column was coupled with a Kontron Instruments SFM 25 fluorescence detector. The linear gradient used was 100% water/acetic acid (5 μL/L) to 100% methanol in 15 min at flow rate of 1 mL/min. Bile samples (10 μL) were directly injected into the liquid chromatographic system, and the chromatograms recorded at 380/430 nm the excitation/emission wavelength pairs of benzo[*a*]pyrene (BaP). Integrated peak areas eluting after 7.5 min (after peak tail of

* Corresponding author phone: 34 93 4006175; fax: 34 93 2045904; e-mail: cpvqam@cid.csic.es.

10.1021/es9902322 CCC: $18.00
Published on Web 07/09/1999

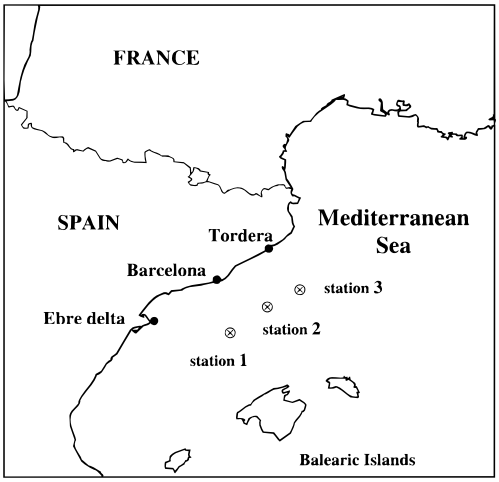

FIGURE 1. Location of the sampling sites: station 1: 40°41′N-1°58′E (1600 m); station 2: 41°01′N-2°34′E (1550 m); and station 3: 41°14′N-3°20′E (1800 m).

tryptophan) in the HPLC chromatograms were summed and quantified as BaP equivalents.

Hydrolysis of Bile and Extraction of Metabolites. Conjugated PAHs metabolites were hydrolyzed by a modification of the method of Krahn et al. (*25*). Briefly, 100 μL of bile were treated with 1 mL of 0.4 M acetic acid/sodium acetate buffer pH 5.0 containing 2000 units of β-glucuronidase and 50 units of sulfatase and incubated for 2 h at 40 °C. Hydrolyzed metabolites were extracted with 1 mL of ethyl acetate (×3), and the extracts were recombined, reduced to 100 μL under nitrogen, and analyzed by gas chromatography−mass spectrometry electron impact mode (GC-MS-EI). Recovery of the extraction procedure was higher than 90% for all the compounds examined (1-naphthol, 2-phenylphenol, 9-fluorenol, and 9-phenanthrol), except for 1-pyrenol which was 85%.

Analysis of Hydrolyzed Bile by GC-MS-EI. Individual quantification of PAHs metabolites in hydrolyzed bile samples was achieved by GC-MS-EI, using a Fisons GC 8000 Series chromatograph interfaced to a Fisons MD800 mass spectrometer. The column, a 30 m × 0.25 mm i.d. HP-5MS cross-linked 5% PH ME siloxane (Hewlett-Packard), was programmed from 80 to 120 °C at 15 °C/min and from 120 to 300 °C at 6 °C/min, keeping the final temperature for 5 min. The carrier gas was helium at 80 kPa. The injector temperature was 250 °C, and the ion source and the analyzer were maintained at 200 and 250 °C, respectively. The mass spectra were obtained at 70 eV by selected ion register (SIR) mode. Metabolites were identified and quantified by comparison of retention times and spectra of reference compounds. Ions used for monitoring were m/z 144, 115 for 1-naphthol; m/z 170, 141 for 2-phenylphenol; m/z 182, 152 for 9-fluorenol; m/z 194, 165 for 9-phenanthrol, and m/z 218, 189 for 1-pyrenol. 2,6-Dibromophenol (m/z 252, 250) and hexamethylbenzene (m/z 162, 147) were used as a surrogate standard and GC internal standard, respectively, and their recoveries were higher than 95%. Detection limits of the GC-MS-EI technique—calculated as signal-to-noise ratio 3:1—were at the low picogram level (4−9 pg), except for 1-naphthol (95 pg) and 1-pyrenol (68 pg).

Preparation of Samples for Biochemical Analysis. Cytosolic and microsomal fractions were prepared essentially as described in Förlin and Andersson (*26*). After weighing, livers were flushed with ice cold 1.15% KCl and homogenized in 4 vol of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 0.1 mM phenyl-methylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500*g* for 10 min, the fatty layer was removed, and the supernatant was centrifuged at 10 000*g* for 20 min. The 10 000*g* supernatant was further centrifuged at 100 000*g* for 60 min to obtain the cytosolic and microsomal fractions. Microsomal pellets were resuspended in a small volume of 100 mM Tris-HCl pH 7.4 containing 0.15 M KCl, 20% w/v glycerol, 1 mM DTT, 1 mM EDTA, and 0.1 mM PMSF.

Cytochrome P450 System. Cytochrome P450 system components were measured in the microsomal fraction as described essentially in Livingstone (*27*). Cytochrome P450 was determined by sodium dithionite-difference spectrum of carbon monoxide treated samples assuming an extinction coefficient of 91 $mM^{-1}\cdot cm^{-1}$ for P450 and 105 $mM^{-1}\cdot cm^{-1}$ for P420 (28). NADPH-cytochrome *c* reductase activity was measured by the increase in absorbance at 550 nm (extinction coefficient 19.6 $mM^{-1}\cdot cm^{-1}$) after NADPH addition to the microsomal fraction (*29*). 7-Ethoxyresorufin *O*-deethylase activity (EROD) was determined at 30 °C as described in Burke and Mayer (*30*); 10 μL of microsomes were incubated for 10 min in a final volume of 1.0 mL containing 90 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.22 mM NADPH, and 3.70 μM 7-ethoxyresorufin. The reaction was stopped by adding 2.0 mL of ice-cold acetone, samples were centrifuged at low speed, and 7-hydroxyresorufin fluorescence was determined using a Perkin-Elmer LS-5 spectrofluorometer at 537/583 nm excitation/emission wavelengths.

Glutathione *S*-Transferase. Glutathione *S*-transferase (GST) activity was measured in the cytosolic fraction using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, with the final reaction mixture containing 1 mM CDNB and 1 mM reduced glutathione (*31*).

Measurement of Proteins. Total biliary proteins as well as microsomal and cytosolic proteins were measured by the method of Lowry et al. (*32*), using bovine serum albumin as a standard.

Statistical Treatment. As insufficient bile was collected from individual fish, bile PAHs metabolites were determined in a pool of 6−10 organisms, analyzed by triplicate, and the results were expressed as mean ± SD. Enzymatic activities and contents were expressed as mean ± SEM of 4−6 organisms analyzed individually. Significant differences were assessed by Student's *t*-test ($p \leq 0.05$).

Results

Biological Data of Samples. Main characteristics of the fish are given in Table 1. Individuals were adults and samples homogeneous (fish size and weight). The condition factor (CF) was higher in *Lepidion lepidion* and *Mora moro*, both organisms from the moridae family. There were no significant differences in terms of liver somatic index (LSI) between sampling sites, though there was a trend toward larger livers in those specimens sampled in station 2. Among species, the largest livers were observed in *C. guentheri* followed by *L. lepidion* and *M. moro*, whereas the lowest LSI was detected in *B. mediterraneus* and *A. rostratus* (0.3−0.6).

PAH Metabolites in Bile. HPLC chromatograms of crude bile recorded at the excitation/emission wavelength pairs of BaP (380/430 nm) showed a complex mixture of fluorescent compounds. Areas of peaks eluting after 7.5 min in the chromatograms were integrated, summed, and quantified as BaP equivalents (Table 2). These results pointed out strong differences between sampling sites. Most of the organisms indicated station 3 as the less polluted in terms of PAHs, whereas station 2, possibly under the influence of anthropogenic inputs from the area of Barcelona, appeared as the most contaminated. Differences among fish species were

TABLE 1. Biological Data of Studied Deep-Sea Fish[a]

	station 1	station 2	station 3
	Weight (g)		
C. guentheri	11.5 ± 1.6 *(6)*	7.1 ± 0.9 *(10)*	7.6 ± 0.7 *(8)*
L. lepidion	60.1 ± 4.3 *(6)*	90.7 ± 15.0 *(6)*	59.3 ± 9.9 *(4)*
M. moro	360.3 ± 45.8 *(6)*	*b*	*b*
B. mediterraneus	18.1 ± 0.6 *(12)*	15.9 ± 0.3 *(12)*	14.0 ± 0.4 *(12)*
A.rostratus	86.8 ± 9.7 *(5)*	153.3 ± 9.2 *(6)*	206.0 *(1)*
	Length (cm)		
C. guentheri	15.9 ± 0.6	12.5 ± 0.6	14.9 ± 0.3
L. lepidion	20.6 ± 0.2	23.9 ± 1.4	21.3 ± 0.7
M. moro	34.6 ± 1.8	*b*	*b*
B. mediterraneus	16.2 ± 0.2	15.1 ± 0.2	15.0 ± 1.1
A. rostratus	24.7 ± 0.9	31.3 ± 0.3	33.0
	CF (g·cm^{-3})		
C. guentheri	0.28 ± 0.02	0.36 ± 0.02	0.25 ± 0.03
L. lepidion	0.70 ± 0.03	0.66 ± 0.03	0.62 ± 0.09
M. moro	0.78 ± 0.02	*b*	*b*
B. mediterraneus	0.42 ± 0.01	0.46 ± 0.01	0.37 ± 0.02
A. rostratus	0.57 ± 0.02	0.50 ± 0.01	0.58
	LSI		
C. guentheri	1.99 ± 0.16	2.45 ± 0.28	1.34 ± 0.12
L. lepidion	1.62 ± 0.34	2.09 ± 0.55	1.07 ± 0.25
M. moro	1.73 ± 0.38	*b*	*b*
B. mediterraneus	0.31 ± 0.05	0.36 ± 0.09	0.29 ± 0.02
A. rostratus	0.32 ± 0.02	0.58 ± 0.06	0.38

[a] CF = condition factor calculated as [(weight)/(length)^3] × 100; LSI = liver somatic index calculated as (liver weight/total weight) × 100. Values are mean ± SEM; number of organisms analyzed per station given in parentheses. [b]Sample not available.

TABLE 2. Biliary Levels of FACs Expressed as BaP Equivalents (ng·mL^{-1}) and Analyzed by HPLC-Fluorescence (380/430 nm)[a]

	station 1	station 2	station 3
C. guentheri	154.4 ± 12.6	198.2 ± 13.3	40.7 ± 5.5
L. lepidion	40.6 ± 3.3	33.2 ± 2.2	31.4 ± 3.0
M. moro	37.8 ± 2.1	*b*	*b*
B. mediterraneus	28.6 ± 2.9	*b*	13.0 ± 1.3
A. rostratus	95.5 ± 8.9	193.9 ± 11.1	15.4 ± 0.9

[a] Each sample is one pool of 6−10 organisms analyzed by triplicate. Results are expressed as mean ± SD. [b]Sample not available.

also evident, the highest levels of FACs were detected in *C. guentheri* and *A. rostratus*. *L. lepidion* was the only fish that did not show differences among sampling sites in terms of FACs.

Interspecies differences were further investigated by analyzing hydrolyzed bile samples of organisms from station 1 by GC-MS-EI (Table 3). In agreement with FACs data, the highest levels of hydroxylated PAHs in bile were detected in *C. guentheri* (501 ng·mL^{-1}), followed by *A. rostratus* (164 ng·mL^{-1}), whereas the rest of species presented lower and similar levels (57−67 ng·mL^{-1}). Looking at the metabolite profile, different PAH patterns were observed. 1-Naphthol, 2-phenylphenol, and 1-pyrenol were equally abundant in *C. guentheri*, and they represented 96% of total detected metabolites. *M. moro* and *L. lepidion* were relatively enriched in 1-naphthol and 2-phenylphenol, which represented 72−85% of total detected metabolites. These two compounds were the only ones detected in *B. mediterraneus*, whereas *A. rostratus* exhibited a different pattern, 1-pyrenol being the major metabolite detected (89% of total metabolites).

Cytochrome P450 System and Glutathione *S*-Transferase. Once taken up by the organisms, PAHs are likely to be subjected to a suite of reactions (monooxygenation and conjugation) that will facilitate their biotransformation and excretion and that may also have an influence on the level of hydroxylated PAHs excreted through the bile. This fact was investigated by measuring xenobiotic metabolizing and conjugating enzymes in four out of five species collected from station 1. The selected species were *C. guentheri*, *L. Lepidion*, *A. rostratus*, and *B. mediterraneus*. *M. moro* was not studied because it belongs to the same family of *L. Lepidion*, and small differences among these two species were observed in terms of PAHs levels or PAH metabolite patterns in bile.

The total cytochrome P450 was determined by dithionite difference spectra of CO-treated microsomes. All the spectra showed a cytochrome P450 peak at 448 nm, and an additional peak at approximately 420−422 nm which may correspond to cytochrome P420. Such apparent degradation varied among species, and it could be linked to the fact that the animals were dead on retrieval, due to the change of pressure and the time needed for collecting the net from 1500 to 1800 m depth (approximately 45 min). Cytochrome P450 specific content was determined in all the species and was found to be higher in *Alepocephalus rostratus* (263 pmol·mg^{-1} protein) (Table 4). Assuming a P420 extinction coefficient of 105 mM^{-1} cm^{-1}, most of the samples contained a greater amount of P420 than P450, and the average values for the relation P420/P450 were 12.2 for *C. guentheri*, 8.4 for *L. Lepidion*, 4.6 for *B. mediterraneus*, and 0.8 for *A. rostratus*. The presence of P420 suggests that the catalytic rates measured here may not reflect the full metabolic capacity of the samples. When combined cytochrome P450 with P420, differences among species were even more evident. The estimate of total P450 content ranged from 1.8 nmol·mg^{-1} protein in *C. guentheri* to 0.48−0.54 nmol·mg^{-1} protein in *A. rostratus* and *B. mediterraneus*. The specific activity of NADPH cytochrome *c* (P450) reductase ranged from 13 to 28 nmol·min^{-1}·mg^{-1} protein (Table 4).

EROD activity was significantly elevated in *L. lepidion* (111 pmol·min^{-1}·mg^{-1} protein) in comparison with the other three species. The lowest EROD activities were detected in *B. mediterraneus* (2.6 pmol·min^{-1}·mg^{-1} protein) and *A. rostratus* (7.7 pmol·min^{-1}·mg^{-1} protein). When EROD activity was expressed as an estimate of turnover number (pmol·min^{-1}·nmol^{-1} P450), *L. lepidion* showed the highest catalytic efficiency (106.1 pmol·min^{-1}·nmol^{-1} P450), followed by *A. rostratus* and *C. guentheri* (28.2 and 13.3 pmol·min^{-1}·nmol^{-1} P450), respectively. The lowest turnover number was recorded for *B. mediterraneus*.

Strong differences among species were also evidenced for the activity of cytosolic GST measured with CDNB as a substrate (Table 4), the highest specific activity detected in *C. guentheri*.

Discussion

Previous work by Lipiatou et al. (*7*) reported that, although 50% of the total PAH inputs in the Western Mediterranean are trapped in the coastal zone (0−200 m depth), nearly 13% of the PAH inputs accumulate in the 1000−2000 m bottom sediments. Considering that hydroxylated PAHs in feral fish reflect levels of exposure, FACs levels recorded in this study indicate that deep-sea regions from the NW Mediterranean are reached by PAHs and that these PAHs are taken up by the organisms from those remote environments. In addition, some fish species, particularly *C. guentheri* and *A. rostratus*, allow us to detect site related differences in terms of FACs (Table 2). Both species indicate that station 3 is the cleanest site, and station 2 is the most polluted. These findings are in agreement with data on LSI index (Table 1), which shows relatively higher values in fish from station 2. Larger livers are generally understood as an indication of chronic or high levels of pollution (*33*); thus, despite its remoteness, deep-sea regions are clearly influenced by anthropogenic inputs from coastal areas. Nevertheless, no site related differences in terms of FACs were evidenced when using *L. lepidion* as

TABLE 3. Individual Hydroxylated PAHs (ng·mL^{-1}) Determined in Hydrolyzed Bile Samples of Different Fish Species from Station 1[a]

	C. guentheri	*L. lepidion*	*M. moro*	*B. mediterraneus*	*A. rostratus*
1-naphthol	124.9 ± 11.5	22.1 ± 3.5	17.8 ± 4.4	30.8 ± 3.1	10.1 ± 3.3
2-phenylphenol	192.7 ± 30.7	32.6 ± 6.0	23.5 ± 4.5	36.2 ± 6.2	8.4 ± 0.9
9-fluorenol	19.9 ± 5.8	nd	nd	nd	nd
9-phenanthrol	nd	nd	nd	nd	nd
1-pyrenol	163.2 ± 41.4	9.8 ± 2.4	16.0 ± 3.4	nd	145.5 ± 25.9
∑PAHs (ng·mL^{-1})	500.7 ± 13.3	64.5 ± 2.4	57.3 ± 2.4	67.0 ± 2.3	164.0 ± 8.7
∑PAHs (ng·mg^{-1} prot)	*42.9 ± 1.1*	*6.6 ± 0.2*	*7.8 ± 0.3*	*15.7 ± 0.5*	*31.1 ± 6.1*

[a] Each sample is a pool of 6−12 organisms analyzed by triplicate; values are mean ± SD. Abbreviation: nd, not detected.

TABLE 4. Cytochrome P450 Dependent Monooxygenase and Glutathione *S*-Transferase Activities in Deep-Sea Fish from NW Mediterranean (Station 1)[e]

	C. guentheri	*L. lepidion*	*B. mediterraneus*	*A. rostratus*
cytochrome P450[a]	137.6 ± 35.9	116.7 ± 18.5	98.0 ± 15.3	262.7 ± 80.1
P450 + P420[a]	1820 ± 173	1093 ± 137	545 ± 119	480 ± 141
NADPH-cit(c) reductase[b]	20.9 ± 3.2	13.1 ± 2.5	27.7 ± 14.2	15.0 ± 2.8
EROD[c]	23.3 ± 3.2	111.2 ± 28.0	2.6 ± 1.2	7.7 ± 1.3
EROD/P450[d]	13.3 ± 4.7	106.1 ± 21.6	5.0 ± 1.1	28.2 ± 8.0
GST[b]	1436 ± 427	983.9 ± 166.3	174.7 ± 102.1	155.4 ± 31.9

[a]pmol·mg^{-1} protein. [b]nmol·min^{-1}·mg^{-1} protein. [c]pmol·min^{-1}·mg^{-1} protein. [d]pmol·min^{-1}·nmol^{-1} P450. [e]Values are mean ± SEM (n = 4−6 specimens).

a sentinel organism. L. lepidion is a middle slope species with a wide distribution (800−1400 m), which feeds actively on mesopelagic fish and euphausiids (34) and, hence, has limited exposure to sediment trapped pollutants.

Generally, FACs levels achieved by deep-sea fish in this study (13−198 ng·mL^{-1} bile) are in the range of those reported for coastal fish (16, 35, 36). Serranus cabrilla, a demersal species that lives in rocky and algae bottoms and is collected in the NW Mediterranean (37), showed levels of FACs in bile (18−224 ng·mL^{-1} bile) similar to those in C. guentheri and A. rostratus (Table 2). In contrast, FACs content in red mullet—Mullus barbatus—from the same area was up to 1 order of magnitude higher (125−1260 ng·mL^{-1} bile) (37). M. barbatus is a benthic-feeder species which lives in contact with the sediment, and the organism-sediment interaction is an important pathway of exposure.

On a qualitative point of view, FACs levels determined at the excitation/emission wavelength pairs of BaP are indicative of the presence of multiring PAHs from combustion sources (25). Types of compounds which fluoresce at BaP wavelengths (380/430 nm) include the following: BaP and its metabolites, selected multiring aromatic compounds and their metabolites (e.g. pyrene, fluoranthene, and its metabolites), and possibly, N-, S-, or O-containing analogues (38). Therefore, GC-MS determination of hydroxylated PAHs in fish bile was carried out as a complementary technique to get information about which particular metabolites are present in the hydrolyzed sample. Nonetheless, the use of the GC-MS technique is limited by the few metabolite standards available and by the need of using derivatization techniques. Attempts were made to silanizate samples and standards, but the sensitivity and accuracy of the method was too low to work with field samples, with low concentrations of PAH metabolites.

On a quantitative point of view, both HPLC-fluorescence and GC-MS indicated low exposure of L. lepidion, M. moro, and B. mediterraneus to PAHs in comparison with C. guentheri and A. rostratus (Tables 2 and 3). Many factors can contribute to the exposure of deep-sea organisms to PAHs and the subsequent detection in fish bile, viz. food web characteristics, habitat, feeding strategies, etc. The feeding status of fish could certainly affect levels of biliary metabolites (10, 39), and, in consequence, methods to establish this status should be developed in order to improve the accuracy of the PAHs analysis. In this study, we have calculated the condition factor (CF) of the analyzed organisms as a general measure of their nutritional status (40). Strong differences among species were observed in terms of the calculated CF (Table 1); however, no clear relationship among levels of PAHs in fish bile and CF could be established. In addition, levels of protein per milliliter of bile were also determined, as it has been reported that the amount of proteins increases markedly in nonfeeding fish (10). B. mediterraneus and A. rostratus, the organisms with a more specialized diet, showed the lowest protein levels (4−5 mg/mL). Nevertheless, when the concentration of proteins was used to normalize residues of hydroxylated PAHs, the observed concentration did not differ strongly from data expressed per milliliter of bile (Table 3).

When looking at the metabolite profile, important differences among species were observed, particularly for 1-pyrenol, that was undetected in B. mediterraneus but represented 89% of the total PAH metabolites determined in A. rostratus. These results are indicative of different patterns of exposure, and again those organisms with a more specialized diet were the ones showing the most particular PAH patterns, namely A. rostratus—a large fish which feeds predominantly on gelatinous macroplankton—and B. mediterraneus—a sedentary fish, well adapted to the oligotrophic deep environment, with low energy requirements, and which feeds on zooplankton carried by the marine currents (41, 42).

Apart from the influence of habitat and trophic strategy, xenobiotic metabolizing enzymes could also have an influence on the level of hydroxylated PAHs excreted through the bile. Hepatic microsomal preparations of the studied organisms showed active electron transport components and native cytochrome P450. Total cytochrome P450 content (P450 + P420) in C. guentheri and L. lepidion were in the higher range (0.9−2.0 nmol·mg^{-1} protein) of the values typically described for teleost species (17, 18). The specific activity of NADPH-cytochrome c (P450) reductase, the microsomal flavoprotein that transfers electrons from NADPH to cytochrome P450, was also high and similar to the activities reported for other species. Therefore, a high monooxygenase capacity of deep-sea fish hepatic tissue can be anticipated. Nevertheless, the detected EROD activities—with the exception of L. lepidion—were in the lower range of those activities usually reported for coastal fish (18). Similarly, the turnover number (EROD activity/P450 content) which is a measurement of the catalytic

efficiency was low in the studied fish, with the exception of L. lepidion that had values similar to coastal fish from the region (23, 37). Nevertheless, it is important to consider that these assays were run in vitro at room temperature and pressure, and the results obtained should therefore be extrapolated with caution to field conditions, where high pressure and cold temperatures may certainly have an influence on membrane functioning, by reducing membrane fluidity and by affecting protein−protein interaction (20). In consequence, the interaction between cytochrome P450 and reductase in the membrane will be less effective in deep-sea fish than in specimens inhabiting shallower waters. In the present study, those organisms better adapted to live at greater depths, namely B. mediterraneus and C. guentheri, showed the lowest EROD turnover, whereas L. lepidion, a typical middle slope species, exhibited the highest turnover.

In addition to characterizing aspects of phase I monooxygenation, glutathione S-transferase, one of the major phase II conjugation enzymes, was also examined. Cytosolic GST activity was in the range or even higher than those activities reported for other fish species (18). The high GST activity detected in C. guentheri correlates with the elevated cytochrome P450 specific content, and this is in accordance with the role of GSTs in conjugating electrophilic compounds produced by P450 monooxygenation. Alternatively, low GST activities were observed in those fish with a more restricted diet (B. mediterraneus and A. rostratus) in comparison with the other two species, which feed actively on epibenthos and infauna (C. guentheri) or large pelagic and benthopelagic prey (L. lepidion) (34). Hence, elevated GST activities may have been selected in those species as protection against toxic dietary chemicals.

Overall, levels of hydroxylated PAHs in bile and activity of xenobiotic metabolizing enzymes in deep-sea fish from the NW Mediterranean are in the range of those observed in fish from shallower waters, directly affected by anthropogenic activities. Despite the number of species and contaminants studied being rather small, the results obtained highlight the usefulness of deep-sea fish as sentinel species to monitor the deep-sea environment. In addition, in view of the importance of deep-sea sediments as the final accumulation site of persistent lipophilic xenobiotics, and the fact that deep-sea sites have been suggested as disposal areas for contaminated wastes, further studies are required to characterize exposure levels of deep-sea fauna to common coastal contaminants as well as the potential negative effects and the final consequences for the whole marine ecosystem.

Acknowledgments

This study was partially supported by the Spanish National Plan for Research (PLANYCIT) under Project Ref. AMB95-1092-CE. We are also grateful to Dr. J. Rucabado and Dr. D. Lloris for their valuable help during samplings and fish identification.

Literature Cited

(1) Woodwell, G. M.; Craig, P. P.; Johnson, H. J. Science **1971**, 174, 1101−1107.
(2) Barber, R. T.; Warlen, S. M. Environ. Sci. Technol. **1979**, 13, 1146−1148.
(3) Krämer, W.; Buchert, H.; Reuter, U.; Biscoito, M.; Maul, D. G.; Le Grand, G.; Walker, C. H. Chemosphere **1984**, 13, 1255−1267.
(4) Fisher, R.; Ballschmiter, K. Fresenius Z. Anal. Chem. **1987**, 327, 42−43.
(5) Berg, V.; Ugland, K. I.; Hareide, N. R.; Aspholm, P. E.; Polder, A.; Skaare, J. U. Mar. Environ. Res. **1997**, 44, 2, 135−148.
(6) Karinen, J. F. Environ. Int. **1980**, 3, 135−144.
(7) Lipiatou, E.; Tolosa, I.; Simó, R.; Bouloubassi, I.; Dachs, J.; Marti, S.; Sicre, M. A.; Bayona, J. M.; Grimalt. J. O.; Saliot, A.; Albaigés, J. Deep-Sea Res. II **1997**, 4, 881−905.
(8) Steimle, F. W.; Zdanowicz, V. S.; Gadbois, D. F. Mar. Pollut. Bull. **1990**, 11, 530−535.
(9) Varanasi, U.; Stein. J. E.; Nishimoto, M. In Metabolism of Polycyclic Aromatic Hydrocarbon in the Aquatic Environment; Varanasi, U., Ed.; CRC Uniscience Series, CRC Press Inc.: Boca Raton, FL, 1989; pp 93−150.
(10) Collier, T. K.; Varanasi, U. Arch. Environ. Contam. Toxicol. **1991**, 20, 462−473.
(11) Britvic, S.; Lucic, D.; Kurelec, B. Environ. Toxicol. Chem. **1993**, 12, 765−773.
(12) Upshall, C.; Payne, J. F.; Hellou, J. Environ. Toxicol. Chem. **1993**, 12, 2105−2112.
(13) Yu, Y.; Wade, T. L.; Fang, J.; McDonald, S.; Brooks, J. M. Arch. Environ. Contam. Toxicol. **1995**, 29, 241−246.
(14) Krahn, M.; Burrows, D. G.; Ylitalo, G. M.; Brown, D. W.; Wigren, C. A.; Collier, T. K.; Chan, S. L.; Varanasi, U. Environ. Sci. Technol. **1992**, 26, 116−126.
(15) Krahn, M.; Ylitalo, G. M.; Buzitis, J.; Bolton, J. L. Mar. Pollut. Bull. **1993**, 27, 285−292.
(16) McDonald, S. J.; Kennicutt II, M. C.; Liu, H.; Safe, S. H. Arch. Environ. Contam. Toxicol. **1995**, 29, 232−240.
(17) Stegeman, J. J.; Hahn, M. E. In Aquatic toxicology: molecular, biochemical, and cellular perspectives; Malins D. C., Ostrander, G. K., Eds.; Boca Raton, FL, 1994; pp 87−204.
(18) Förlin, L.; Lemaire, P.; Livingstone, D. R. Mar. Environ. Res. **1995**, 39, 201−204.
(19) Gibbs, A. G. In Deep-sea fishes; Academic Press: 1997; pp 239−277.
(20) Gross, M.; Jaenicke, R. Eur. J. Biochem. **1994**, 227, 617−630.
(21) Stegeman, J. J.; Kloepper-Sams, P. J.; Farrington, J. W. Science **1986**, 231, 1287−1289.
(22) Porte, C.; Albaigés, J. Arch. Environ. Contam. Toxicol. **1993**, 26, 273−281.
(23) Burgeot, T.; Bocquéné, G.; Porte, C.; Dimeet, J.; Santella, R. M.; García de la Parra, L. M.; Pfhol-Leszkowicz, A.; Raoux, C.; Galgani, F. Mar. Ecol. Prog. Ser. **1996**, 131, 125−141.
(24) Krahn, M.; Myers, M. S.; Burrows, D. G.; Malins, D. C. Xenobiotica **1984**, 14, 633−646.
(25) Krahn, M.; Burrows, D. G.; MacLeod, W. D., Jr.; Malins, D. C. Arch. Environ. Contam. Toxicol. **1987**, 16, 511−522.
(26) Förlin, L.; Anderson, T. Comput. Bichem. Physiol. **1985**, 80B, 569−572.
(27) Livingstone, D. R. Mar. Ecol. Prog. Ser. **1988**, 46, 37−43.
(28) Estabrook, R. W.; Werringloer, J. In Methods of Enzymology, vol. 52 Biomembranes: Part C: Biological Oxidations, Microsomal, Cytochrome P-450, and Other Hemoprotein Systems; Fleischer, S., Packer, L., Eds.; Academic Press: New York, 1978; pp 212−220.
(29) Shimakata, T.; Mihara, V.; Sato, R. J. Biochem. **1972**, 72, 1163−1174.
(30) Burke, M. D.; Mayer, R.T. Drug Metab. Disp. **1974**, 2, 583−588.
(31) Habig, W. H.; Pabst, M. J.; Jakoby, W. B. J. Biol. Chem. **1974**, 25, 7130−7139.
(32) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. J. Biol. Chem. **1951**, 193, 265−275.
(33) Sloof, W.; Van Kreijl, C. F.; Baars, A. J. Aquat. Toxicol. **1983**, 4, 1−14.
(34) Stefanescu, C.; Rucabado, J.; Lloris, D. Mar. Ecol. Prog. Ser. **1992**, 81, 205−213.
(35) McCain, B. B.; Malins, D. C.; Krahn, M. M.; Brown, D. W.; Gronlund, W. D.; Moore, L. K.; Chan, S. L. Arch. Environ. Contam. Toxicol. **1990**, 19, 10−16.
(36) Myers, M. S.; Stehr, C. M.; Olson, O. P.; Johnson, L. L.; McCain, B. B.; Chan, S. L.; Varanasi, U. Environ. Health Perspec. **1994**, 102, 2, 200−215.
(37) Escartín, E. Ph.D. Thesis, Universitat de Barcelona, 1999; 269 pp.
(38) Krahn, M. M.; Rhodes, L. D.; Myers, M. S.; Moore, L. K.; MacLeod, W. D.; Malins, D. C. Arch. Environ. Contam. Toxicol. **1986**, 15, 61−67.
(39) Brumley, C. M.; Haritos, V. S.; Ahokas, J. T.; Holdway, D. A. Ecotox. Environ. Saf. **1998**, 39, 147−153.
(40) Bagenal, T. B.; Tesch, F. W. In Age and Grow; Bagenal, T. B., Ed.; Blackwell Scientific Publications: Oxford, 1978; pp 101−136.
(41) Morales-Nin, B.; Massutí, E.; Stefanescu, C. J. Fish Biol. **1996**, 48, 1097−1112.
(42) Stefanescu, C.; Lloris, D.; Rucabado, J. Deep-sea Res. I **1993**, 40, 695−707.

Received for review February 26, 1999. Revised manuscript received May 24, 1999. Accepted May 26, 1999.

ES9902322