

Assessment of coastal pollution by combined determination of chemical and biochemical markers in *Mullus barbatus*

Cinta Porte*, Estefania Escartín, Luz M. García de la Parra**, Xavier Biosca, Joan Albaigés

Department of Environmental Chemistry, IIQAB-CSIC, Jordi Girona 18–26, 08034 Barcelona, Spain

ABSTRACT: Muscle concentrations of organochlorinated compounds as well as biliary levels of polycyclic aromatic hydrocarbon (PAH) metabolites were determined in red mullet *Mullus barbatus* as a sentinel species for assessing the pollution along the western Mediterranean coast. A battery of biochemical markers—biotransformation and antioxidant enzymes—were also measured in liver sub-cellular fractions to assess exposure to pollutants. Among them, 7-ethoxyresorufin *O*-deethylase (EROD) activity, as well as 7-penthoxyresorufin *O*-deethylase (PROD) activity and cytochrome P450 1A (CYP1A) content evidenced strong differences among sampling sites, and a good correlation with the amount of PCBs bioaccumulated by fish. No clear pollution-related response was observed for cytosolic glutathione *S*-transferase, whereas uridine-diphosphate UDP-glucuronyltransferase (UDPGT) was elevated in fish from polluted sites. Antioxidant enzymes—superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX)—were used to assess oxidative stress. Among them, catalase activity was well related to PCB body burden.

KEY WORDS: Cytochrome P450 system · Antioxidant enzymes · Biomonitoring · *Mullus barbatus*

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Fishes inhabiting coastal areas have been proposed as sentinels for monitoring land-based pollution because they may concentrate indicative hydrophobic compounds in their tissues, directly from water through respiration and also through their diet. Once in the organism, these xenobiotic compounds undergo a series of biotransformation reactions catalyzed by different enzymatic systems, the activation of which may also be an additional evidence of pollution exposure (Walker & Livingstone 1992). Therefore, the comprehensive determination of chemical and biochemical markers in coastal fishes is a sound procedure for

assessing continental pollutant inputs into these areas. The present study was intended to provide new insights into this approach.

The western Mediterranean was selected for the study because it is the recipient of extensive urban and industrial waste-water discharges from bordering countries, and at the same time is an attractive leisure region; it is thus in urgent need of tools for environmental risk assessment. Most of the present data available refers to biota levels of xenobiotics, such as metals, polycyclic aromatic hydrocarbons (PAHs), organophosphorus pesticides and organochlorinated compounds (Albaigés et al. 1987, Porte & Albaigés 1993, Pastor et al. 1996, Baumard et al. 1998). However, information on the fate and toxicity of these and other chemicals that are currently released into the marine environment is scarce, and very few monitoring programs have addressed the integrated use of chemical analyses with biochemical and cellular responses to pollutants (Romeo et al. 1994, Burgeot et al. 1996, Porte et al. 2001).

*E-mail: cpvqam@cid.csic.es

**Present address: Centro de Investigación en Alimentación y Desarrollo, A.C. Unidad Mazatlán en Acuicultura y Manejo Ambiental, Avenida Sábalo-Cerritos s/n, 82010 Mazatlán, Sinaloa, Mexico

The red mullet *Mullus barbatus* was chosen as bio-indicator species because it is a benthic and territorial fish of commercial interest in the region, which has been used in several studies of coastal pollution monitoring (Porte & Albaigés 1993, UNEP 1997). Fish were collected from 10 stations along the Spanish, French, Corsican and Sardinian coasts, to provide a wide coverage of the area and encompass large pollution gradients. The organic contaminants considered were PAHs and organochlorinated compounds, both ubiquitous in the area (Tolosa et al. 1995, 1996). The cytochrome P450 monooxygenase system, namely, total cytochrome P450, NADPH-cytochrome (P450) *c* reductase, 7-ethoxyresorufin and 7-penthoxyresorufin *O*-deethylase (EROD and PROD), and CYP1A content were determined in liver microsomal fractions. Glutathione *S*-transferases (GST) and UDP-glucuronyl transferases (UDPGT) were determined as Phase II enzymes. Additionally, since many pollutants in aquatic ecosystems exert their toxic effects through oxidative stress (di Giulio et al. 1989, Schlezinger & Stegeman 2001), the antioxidant enzymes (catalase, superoxide dismutase [SOD] and glutathione peroxidase [GPX]) were also determined as a measure of defence mechanisms against oxidative damage. These enzymes have been detected in a number of fish species (Lemaire & Livingstone 1993), but their incorporation in biomonitoring programs is still controversial.

MATERIALS AND METHODS

Sample collection and preparation. Red mullets *Mullus barbatus* were collected by trawling at 30 to 50 m depth in 2 surveys carried out in April 1995 (Catalan coast, Spain) and October 1995 (Gulf of Lions, Corsica and Sardinia) (Fig. 1). Fish were killed on board by severing the spinal cord. The liver was dissected immediately, frozen in liquid nitrogen and stored at -80°C . The gall bladder was removed and stored in dark vials at -20°C . A piece of dorsal muscle tissue (5 g) was also dissected, wrapped in aluminium foil and stored at -20°C for analysis of organochlorinated compounds.

Chemical analysis. The analytical methods were as follows:

Organochlorinated compounds (OCs): Muscle samples of 4 to 6 individuals per location were pooled and subsamples of approximately 4 to 5 g were homogenised with anhydrous Na_2SO_4 and Soxhlet-extracted with *n*-hexane:dichloromethane (4:1) for 18 h. The solvent extract was evaporated to near dryness, the residue dissolved in 3 ml of *n*-hexane and cleaned up by vigorous shaking with 1 to 2 ml of concentrated H_2SO_4 . The resultant extract was washed with water,

dried over Na_2SO_4 and diluted with *n*-hexane for GC-ECD analysis. The instrument was a Hewlett Packard 5890 GC equipped with an ECD detector. The column, a 50 m \times 0.25 mm i.d. CP-Sil 5 CB fused silica (Chrom-pack), was programmed from 80 to 180°C at $15^{\circ}\text{C min}^{-1}$ and from 180 to 280°C at $3^{\circ}\text{C min}^{-1}$ maintaining the final temperature for 15 min. The carrier gas was helium at a linear flow-rate of 50 cm s^{-1} . The injector and detector temperatures were set at 280 and 300°C , respectively. Quantitation was performed using an external standard calibration mixture of selected congeners (IUPAC Nos.: 28, 52, 101, 118, 138, 153, 180) supplied by Promochem (Wesel, Germany) and recommended by the International Council for the Exploration of the Sea (ICES) for assessing marine pollution (Duinker et al. 1988). These congener were quantified separately and the PCB concentration defined as its sum. *p,p'* DDT and its metabolites *p,p'* DDE and *p,p'* DDD and hexachlorobenzene (HCB) were also determined.

Selected samples (1 per tissue) were analysed for confirmatory identifications as described by Porte & Albaigés (1993) following a method involving fractionation of the organic extracts, and gas chromatography-mass spectrometry (GC-MS) analysis in the negative ion chemical ionization mode.

Hydroxylated-PAHs in fish bile: Bile samples were hydrolysed by a modification of the method of Krahn et al. (1987) as described in Escartín & Porte (1999). Briefly, 100 μl of bile were incubated for 2 h at 40°C in the presence of 1 ml 0.4 M acetic acid/sodium acetate buffer, pH 5.0, containing 2000 units of β -glucuronidase and 50 U of sulphatase, and 40 ng of 2,6-dibromophenol as a surrogate standard. Hydrolysed metabolites were extracted thrice with 1 ml ethyl acetate. An aliquot of this extract was analysed by GC-MS after the addition of 20 ng hexamethylbenzene as internal standard. The equipment was a Fisons GC 8000 Series chromatograph interfaced to a Fisons MD800 mass spectrometer. The column, a 30 m \times 0.25 mm i.d. HP-5MS crosslinked 5% PH ME siloxane (Hewlett-Packard) was programmed from 80 to 120°C at $15^{\circ}\text{C min}^{-1}$ and from 120 to 300°C at $6^{\circ}\text{C min}^{-1}$, maintaining the final temperature for 5 min. The injector temperature was 250°C , and the ion source and the analyser were maintained at 200 and 250°C , respectively.

The metabolites were identified and quantified by comparison of retention times and spectra of reference compounds. The ions used for monitoring were: *m/z* 144 and 115 for 1-naphthol; *m/z* 170 and 141 for 2-phenylphenol; *m/z* 182 and 152 for 9-fluoreno; *m/z* 194 and 165 for 9-phenanthrol; and *m/z* 218 and 189 for 1-pyrenol.

Analytical performance: The above methods included the processing of blanks, duplicates and standard mixtures for each group of samples. The GC

injections were performed with an automatic injector to improve reproducibility; this was <14% for the whole procedure. The recoveries calculated from the surrogate standards were >90%. The detection limits were 0.01 to 0.07 ng g⁻¹ for DDT and PCB congeners in muscle, and 0.04 to 0.09 ng ml⁻¹ for hydroxylated PAHs in bile, except for 1-naphthol and 1-pyrenol (0.7 to 0.9 ng ml⁻¹). The protocol for determination of organochlorinated compounds was validated through the participation in intercalibration exercises (United Nations Environment Programme-Intergovernmental Oceanographic Commission-International Atomic Energy Agency, UNEP-IOC-IAEA).

Biochemical determinations. Cytosolic and microsomal fractions were prepared essentially as described in Förlin & Andersson (1985). After weighing, livers were flushed with ice-cold 1.15% KCl and homogenised in 1:4 w/v of cold 100 mM KH₂PO₄/K₂HPO₄ buffer, pH 7.4, containing 0.15 M KCl and supplemented with 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 obtained 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 and supplemented with 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. Cytosolic and microsomal proteins were measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Cytochrome P450 system: Cytochrome P450 system components were measured in the microsomal fraction. Cytochrome P450 was determined from the sodium dithionite-difference spectrum of carbon monoxide-treated samples, assuming an extinction coefficient of 91 mM⁻¹ cm⁻¹ (Estabrook & Werringloer 1978), NADPH-cytochrome *c* reductase activity was measured by the increase in absorbance at 550 nm (extinction coefficient 19.6 mM⁻¹ cm⁻¹) after NADPH addition to the microsomal fraction (Shimakata et al. 1972). 7-ethoxyresorufin *O*-deethylase (EROD) and 7-penthoxyresorufin *O*-deethylase (PROD) activities were determined at 30°C using 10 and 60 min incubation times, respectively, essentially as described in Burke & Mayer (1974) and Lubet et al. (1985); 10 µl of microsomes (EROD) or 100 µl (PROD determination) were incubated in a final volume of 1.0 ml containing 90 mM KH₂PO₄/K₂HPO₄, pH 7.4, 0.22 mM NADPH and 3.8 µM 7-ethoxyresorufin or 7-penthoxyresorufin. The reaction was stopped by adding 2.0 ml of ice-cold acetone.

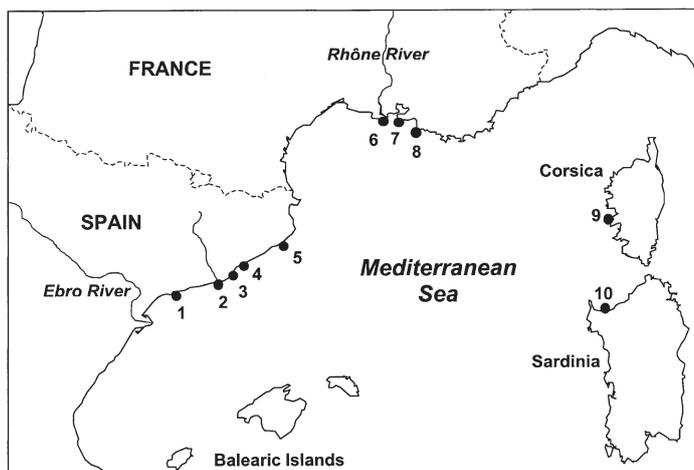


Fig. 1. Map of the Mediterranean Sea showing the sites where red mullet *Mullus barbatus* was sampled. 1: Tarragona; 2: Llobregat River; 3: Barcelona; 4: Besós River; 5: Tordera River; 6: Rhône Delta; 7: Couronne; 8: Cortiou (Marseilles); 9: Cap Feno; 10: Porto Bay

Samples were centrifuged at low speed, and 7-hydroxyresorufin fluorescence was determined using a Kontron Instruments SFM-25 spectrofluorimeter at 537/583 nm excitation/emission wavelengths.

Immunodetection of cytochrome P4501A: Microsomal fractions were thawed and samples of equivalent total protein content (2.5 mg ml⁻¹) were boiled for 5 min in SDS-PAGE buffer (Laemmli 1970) and loaded for separation onto 10% polyacrylamide gels topped by 3% polyacrylamide stacking gels. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham, UK) using a buffer containing 150 mM glycine in 20 mM Tris and 20% methanol (v/v). Transfer was carried out for 30 min at room temperature using a Trans-Blot semi-dry cell (Bio-Rad). The nitrocellulose membranes were then blocked for 30 min in Tris-buffered saline (TBS) pH 8.0, which contained 0.2% Tween 20, 0.5% gelatine and 0.1% sodium azide, and subsequently probed using a mouse anti-cod CYP1A1 monoclonal antibody (Biosense Laboratories, Norway), and anti-mouse IgG alkaline phosphatase conjugated as a secondary antibody. Alternatively, a number of samples were probed using a rat Cytochrome P4501A1-ECL Western blotting kit (Amersham, UK). Comparisons between blots were facilitated by including an internal control (β -naphthoflavone-induced rainbow trout) on every blot. Blots were semi-quantified using a laser densitometer (Epson GT-8000), and the absorbance of the samples expressed as percentages of the standard.

Phase II enzymes: Glutathione *S*-transferase activity was measured in the cytosolic fraction using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al.

1974), the final reaction mixture containing 1 mM CDNB and 1 mM-reduced glutathione. UDPGT activity was assayed by a modification of the method of Clarke et al. (1992). The assay, which contained 0.25 mg microsomal protein (pretreated for 5 min with 0.2% Triton X-100 on ice), was initiated by the addition of 80 μ M *p*-nitrophenol (*p*-NP) and run for 30 min at 30°C in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloroacetic acid, followed by centrifugation and alkalisation with 0.1 ml 10 M KOH; the remaining *p*-NP was measured spectrophotometrically at 405 nm.

Antioxidant enzymes: Antioxidant enzymes were measured essentially as described in Livingstone et al. (1992). Catalase activity was measured in the cytosolic fraction (100 000 \times *g* supernatant) and in the 12 000 \times *g* pellet after the latter had been resuspended in a small volume of homogenisation buffer. This activity was measured by the decrease in absorbance at 240 nm (extinction coefficient 40 M⁻¹ cm⁻¹) using 50 mM H₂O₂ as a substrate, and expressed as the sum of both fractions' activities. SOD activity was determined by the degree of inhibition of cytochrome *c* reduction by superoxide anion radical, measuring the absorbance at 550 nm as described in McCord & Fridovich (1969). The activity of the enzyme given in SOD units (1 unit = 50% inhibition of the cytochrome *c* reduction). GPX activity was measured by the NADPH consumption monitored at 340 nm (extinction coefficient 6.2 mM⁻¹ cm⁻¹) during the formation of reduced glutathione by commercial glutathione reductase (Günzler & Flohé 1985), using 0.8 mM H₂O₂ as a substrate (Se-dependent GPX) or 3 mM cumene hydroperoxide (sum of Se-dependent and Se-independent activities and referred as a total GPX).

Statistical procedures: Biochemical activities were determined individually in 4 to 6 organisms per station and run in duplicate. Values are presented as means \pm SEM. Statistical significance was assessed using

1-way ANOVA with a Duncan's test. A *p*-value of <0.05 was considered as statistically significant. Pearson's correlation coefficients were calculated and only *p* < 0.05 was accepted as significant.

RESULTS

Biological data of samples

The biological parameters of the sampled fish are listed in Table 1. All individuals were adults and similar in size and weight, except those from Stn 2 which were smaller (*p* < 0.05). The liver somatic index (LSI) was significantly higher in organisms sampled in April (Stns 1 to 5) than in October (Stns 6 to 10). The LSI values found in organisms from the first sampling ranged between 2.1 and 2.7%, with the exception of organisms from Stns 2 (1.8%). In the second sampling, LSI values were in the range of 0.9 to 2.0%. The condition factor (CF) was quite similar (*p* > 0.05) in all individuals sampled (1.2 to 1.6), indicating a similar nutritional state of the studied fish.

Bioaccumulation of xenobiotics

Polychlorinated biphenyls (PCBs)

The GC-ECD profiles of the fish tissue extracts showed the occurrence of a series of PCB congener with 3 to 8 chlorine substituents. Representative distributions, normalised to Congener No. 153, are shown in Fig. 2. They exhibited the usual predominance of Congener Nos. 153, 138, 187, 180 and 170, all with chlorine substitutions in the Positions 2, 4 and 5 of 1 or 2 aromatic rings, and known to be particularly refractory to metabolic degradation (Muir et al. 1988). A common feature was also the higher predominance of low chlo-

Table 1. *Mullus barbatus*. Biological data of red mullets sampled in April (Stns 1 to 5) and October 1995 (Stns 6 to 10). *Significant differences from the reference site (Stn 5); n: number of organisms analysed; LSI: liver somatic index ([liver weight/body weight] \times 100); CF: condition factor ([body weight/length³] \times 100)

Station	n	Weight (g)	Length (cm)	LSI (%)	CF (g cm ⁻³)
1: Tarragona	6	81.3 \pm 8.4	17.3 \pm 0.6	2.60 \pm 0.24	1.60 \pm 0.20
2: Llobregat River	4	23.7 \pm 8.8*	11.7 \pm 1.4	1.80 \pm 0.45	1.37 \pm 0.09
3: Barcelona	6	70.2 \pm 6.5	16.8 \pm 0.5	2.66 \pm 0.14	1.46 \pm 0.06
4: Besós River	6	76.9 \pm 16.6	16.8 \pm 1.1	2.40 \pm 0.27	1.50 \pm 0.06
5: Tordera River	6	40.6 \pm 5.4	14.4 \pm 0.6	2.10 \pm 0.20	1.30 \pm 0.12
6: Rhône Delta	5	76.7 \pm 21.2	16.9 \pm 2.1	2.00 \pm 0.41	1.31 \pm 0.03
7: Courone	4	61.2 \pm 20.8	15.8 \pm 2.2	1.01 \pm 0.13	1.22 \pm 0.07
8: Cortiou	6	98.7 \pm 10.3	20.1 \pm 0.7	1.51 \pm 0.13	1.20 \pm 0.10
9: Cap Feno	4	67.0 \pm 3.5	17.5 \pm 0.4	1.44 \pm 0.07	1.25 \pm 0.01
10: Porto Bay	6	51.7 \pm 2.5	15.3 \pm 0.3	0.93 \pm 0.11	1.45 \pm 0.11

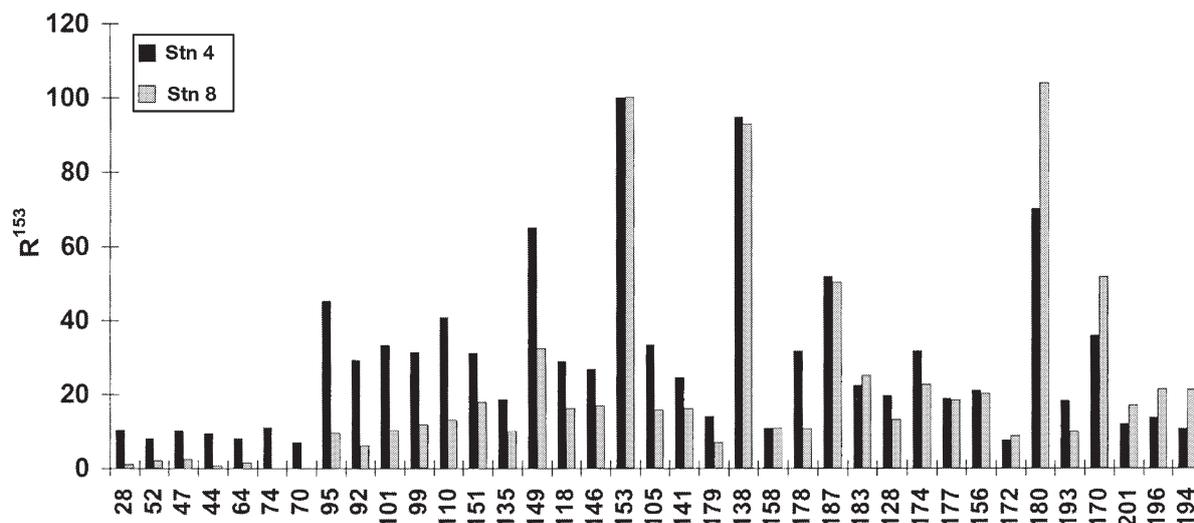


Fig. 2. *Mullus barbatus*. Bioaccumulation profiles of individual polychlorinated biphenyls, related to Congener No. 153, in tissues of red mullet collected in areas under influence of Barcelona (Stn 4) and Marseille (Stn 8). PCB-153 concentration was 71.3 and 157.2 ng g⁻¹ wet wt for Stns 4 and 8, respectively; PCB concentration was 215 and 450 ng g⁻¹ wet wt, respectively

minated congener in the samples collected from the Spanish coast relative to those from the French coast. This was particularly noticeable for the pentachlorinated congener (e.g. Nos. 95, 92, 101, 99 and 110).

These differences were more apparent when the global distributions of the identified congeners were considered. The distributions of tri- to octachlorobiphenyls of selected stations are summarised in Fig. 3. Hexachlorobiphenyls represented 37 to 46% of the whole mixture, followed by the hepta- (18 to 39%) and pentachlorobiphenyls (10 to 27%), the mullets from the French coast exhibiting relatively higher levels of hepta- and octachlorobiphenyls.

PCB residues were quantified on the basis of the 7 PCB congener recommended by ICES for assessing marine pollution (Duinker et al. 1988); this also enabled their comparison with data obtained in previous studies in the region. As shown in Table 2, the highest PCB levels were in the area of influence of Barcelona (Stns 3 and 4) and Marseille (Stn 8), whereas the lowest were in fish from the Corsica and Sardinia Islands (Stns 9 and 10), which can be considered reference sites. Intermediate values were found for other stations along the Spanish and French coasts.

Organochlorinated pesticides

The major components identified in the fish tissues were HCB, p,p' DDT and their main metabolites p,p' DDD and p,p' DDE. The levels of total DDTs are shown in Table 2. The spatial pattern was similar to

that exhibited by PCBs, although the red mullet concentrations from the Spanish coast, and particularly from the area of Barcelona (Stns 3 and 4), were much higher, with levels up to 230 ng g⁻¹ wet wt. This difference was mainly due to a higher contribution of p,p' DDE to total DDTs. In fact, the p,p' DDT concentration in fish from the area of Marseille was about twice that in fish from Barcelona but, as shown in Table 2, the DDE/DDT ratios were 1 order of magnitude higher in the latter. Considering that all organisms exhibited a similar metabolic activity, this high concentration of p,p' DDE in the tissue of fish from Barcelona is attributed to a specific input of the compound, a subproduct

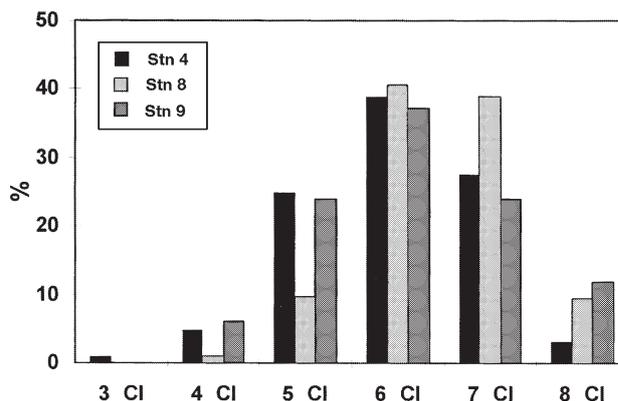


Fig. 3. Distribution of PCB congeners (abscissa) in muscular tissue according to their chlorine (CL) substitution at selected stations. PCB concentrations were 215 ng g⁻¹ wet wt (Stn 4), 450 ng g⁻¹ wet wt (Stn 8), and 1.7 ng g⁻¹ wet wt (Stn 9)

Table 2. *Mullus barbatus*. Organochlorinated pollutants (PCBs, DDTs and HCB) in muscle and hydroxylated PAHs in red mullet bile. All compounds determined from pool of 4 to 6 organisms and expressed as ng g⁻¹ wet wt (organochlorinated compounds) and ng ml⁻¹ (hydroxylated PAHs). -: not determined

Station	Σ PCBs ^a	Σ DDTs ^b	DDE/DDT	HCB	Σ OH-PAHs ^c
1: Tarragona	84	34	11	2.8	165
2: Llobregat River	65	82	24	1.5	-
3: Barcelona	200	230	26	1.5	1590
4: Besós River	215	220	22	1.9	-
5: Tordera River	23	16	5	0.2	-
6: Rhône Delta	60	11	1	0.7	125
7: Courone	61	13	1	1.0	90
8: Cortiou	450	35	0.8	0.4	480
9: Cap Feno	1.7	0.8	2	0.5	-
10: Porto Bay	2.3	0.6	2	0.8	150

^aSum of Congener Nos. 28, 52, 101, 118, 153, 138, 180
^bSum of p,p' DDE, p,p' DDD and p,p' DDT
^cSum of 1-naphthol, 2-phenylphenol, 9-fluorenel, 9-phenanthrol, and 1-pyrenol

in the synthesis of the pesticide dicofol (Ballschmiter & Wittlinger 1991), which is produced in the area.

Measurable amounts of HCB were also found in all samples (Table 2), with levels along the Spanish coast being slightly higher than those along the French coast. This is probably related to the existence of an

industrial source in the vicinity of Stn 1, upstream of the Ebro River (Grimalt et al. 1988).

Polycyclic aromatic hydrocarbons

Aromatic hydrocarbon exposure in marine organisms has often been assessed by measuring the concentrations of PAHs in their tissues either by UV-fluorescence (Albaigés et al. 1987, Porte & Albaigés 1993) or, more recently, by GC-MS (Baumard et al. 1998). However, fishes often show only trace levels of PAH in their tissues, due to their ability to metabolise these compounds (Varanasi et al. 1989). Thus, an alternative technique for assessing PAH exposure is the determination of

PAHs excreted through the bile as conjugated metabolites. Laboratory and field studies have both demonstrated that the presence of PAH metabolites in bile is well correlated with levels of exposure (Collier & Varanasi 1991, Britvic et al. 1993, Upshall et al. 1993, Yu et al. 1995).

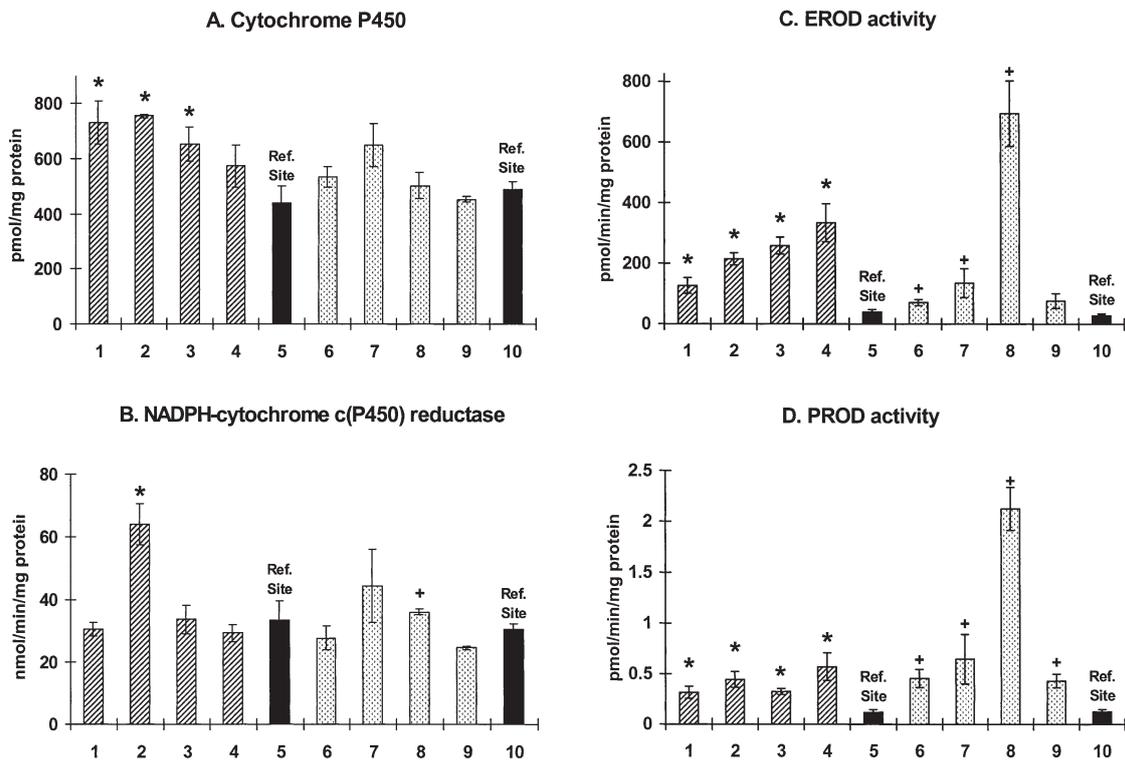


Fig. 4. *Mullus barbatus*. Levels and activities of Phase I-related enzymes in liver of red mullet sampled along the NW Mediterranean coast (sites on abscissa, numbered as in Fig. 1). Each value is mean \pm SEM (n = 4 to 6). *Significant difference from reference site at Stn 5; +: significant difference from reference site at Stn 10

The values in Table 2 correspond to the sum of individual components identified and determined by GC-MS (Escartin & Porte 1999), namely: 1-naphthol, 2-phenylphenol, 9-fluorene, 9-phenanthrene and 1-pyrene.

Biochemical responses

Cytochrome P450 system

Total cytochrome P450 in hepatic microsomal preparations of *Mullus barbatus* ranged between 441 and 755 pmol mg⁻¹ protein. During the first cruise, P450 content was significantly elevated ($p \leq 0.05$) in fish from Stns 1 to 3 compared with the reference site (Stn 5; Fig. 4A). During the second cruise, no significant differences between stations were observed in terms of cytochrome P450 content, despite the high levels of PCBs (450 ng g⁻¹ wet wt) or hydroxy-PAHs (480 ng ml⁻¹) recorded in specimens from Cortiou. NADPH cytochrome (P450) c reductase activity ranged between 24 and 64 nmol min⁻¹ mg⁻¹ protein. Statistically significant differences were observed in fish from Stns 2 and 8 compared with their respective reference sites (Fig. 4B). No clear relationship between pollutant exposure and reductase activity was observed.

In contrast, EROD activity revealed clear pollutant gradients (Fig. 4C). The highest activity was recorded in red mullets from Stn 8, influenced by the urban and industrial area of Marseille. EROD activity was 25-fold higher than in organisms from Stn 10 (reference site). High EROD activity (5- to 8.5-fold higher than at the reference site) was also recorded in organisms from the area of influence of Barcelona (Stns 2 to 4). The PROD activity profile (Fig. 4D) was similar, but differences between stations were smaller.

P4501A content in fish from the first cruise was determined by 2 different antibody detection methods: (1) anti-rat cytochrome P4501A1 ECL (enhanced chemiluminescence) detection, and (2) anti-cod cytochrome P4501A1/alkaline phosphatase detection. A protein cross-reacting with monoclonal anti-rat CYP1A1 IgG was observed in all samples except those from Stn 5 (reference site). This cross-reacting protein was an intense single band with a molecular weight of 58 kDa, coinciding with positive control, β -naphthoflavone-induced rainbow trout. With the second method, a protein cross-reacting with monoclonal anti-cod CYP1A1 IgG was observed in all samples, including those from the reference site. Western blots were quantified by density-image analysis, and the results are shown in Fig. 5. A good correlation between values calculated by the 2 methods was found for P4501A ($r^2 = 0.67$; $p = 0.008$), as well as for EROD activity, particularly with

monoclonal anti-cod CYP1A1 ($r^2 = 0.77$; $p = 0.002$). Additionally, this antibody had higher reproducibility and less non-specific binding; therefore, it was chosen for the analysis of samples from the second cruise (Fig. 6). The CYP1A content was significantly high in Stns 1 to 4 and 7 to 8, and in good agreement with microsomal EROD activity ($r^2 = 0.73$; $p = 0.010$), but not with PROD activity ($r^2 = 0.33$; $p = 0.065$).

Phase II enzymes

The activity of cytosolic GST toward CDNB was variable (82 to 638 nmol min⁻¹ mg⁻¹ protein) and showed no consistent relationship with pollutant body-burden (Fig. 7A). In fact, during the first cruise the highest GST was recorded in fish from the reference site (Stn 5), whereas during the second cruise the lowest GST was observed for the reference site (Stn 10), and fish from the most polluted sites (Stns 6 and 8) had significantly higher GST activities ($p < 0.05$). In contrast, UDPGT activity determined in the microsomal fraction achieved minimum levels in organisms from reference sites (0.30 and 0.37 nmol min⁻¹ mg⁻¹ protein at Stns 5 and 10, respectively), and significantly higher activi-

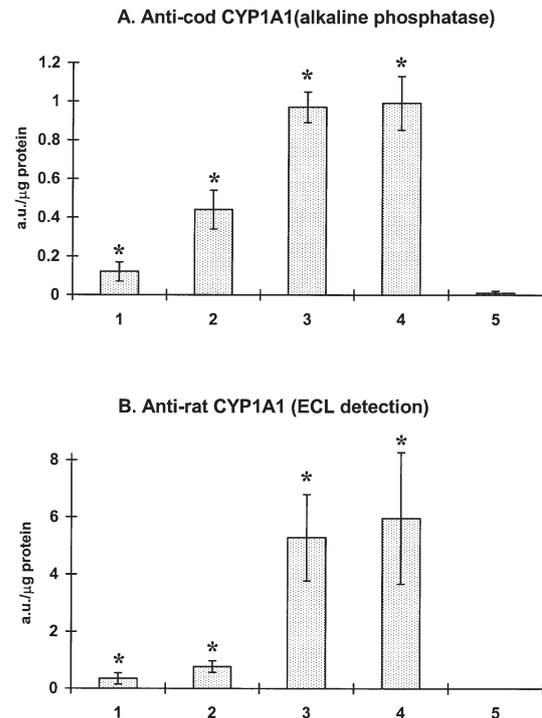


Fig. 5. *Mullus barbatus*. Comparison of CYP1A1 levels in red mullet sampled along the Catalan coast (sites on abscissa, numbered as in Fig. 1) determined by 2 different methods and antibodies (a.u.: arbitrary units). Each value is mean \pm SEM ($n = 4$ to 6). * Significant difference from reference site at Stn 5

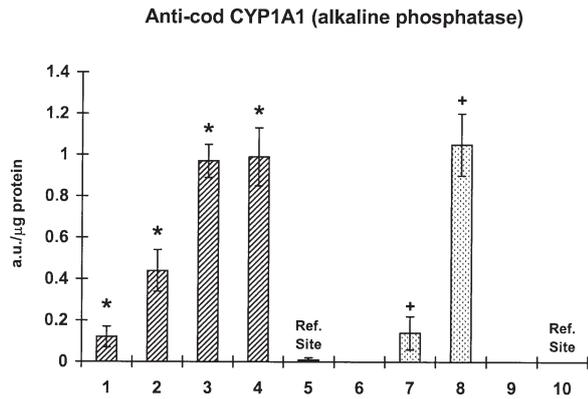


Fig. 6. *Mullus barbatus*. CYP1A levels in red mullet sampled along the NW Mediterranean coast (sites on abscissa, numbered as in Fig. 1) analysed by the alkaline phosphatase method (a.u.: arbitrary units). Each value is mean \pm SEM (n = 4 to 6). *Significant difference from reference site at Stn 5; +: significant difference from reference site at Stn 10

ties at Stns 1, 7 and 8 (0.53, 0.59 and 0.65 nmol min⁻¹ mg⁻¹ protein, respectively: Fig. 7B).

Antioxidant enzymes

Antioxidant enzyme activities (catalase, SOD, GPX) are presented in Fig. 8. Catalase activity was significantly elevated in organisms from Stns 1, 3, 4 and 8 (59 to 64 mmol min⁻¹ mg⁻¹ protein) in comparison with the reference sites (32 to 34 mmol min⁻¹ g⁻¹ wet wt). SOD activity ranged between 4.6 and 14.4 U mg⁻¹ protein (Fig. 8B). Significant differences among sampling sites were recorded, but had no clear relationship to the levels of bioaccumulated pollutants. In contrast, no pollution- or site-related differences were observed for GPX activities (Se-dependent and total: Fig. 8C,D). Maximum activities were recorded for the 2 reference sites, but differences among sites were not statistically significant.

DISCUSSION

Xenobiotics

The residue analysis of red mullet tissues has evidenced that the organochlorinated pollutants are ubiquitous in the region. However, significant differences, both qualitative and quantitative, were found among samples. Fig. 3 shows that mullets from the French coast (e.g. Stn 8) exhibited relatively higher levels of hepta- and octachlorobiphenyls than those from the Spanish coast (e.g. Stn 4), which were enriched in tetra- and pentachlorinated congener. Similar differences were also found in sediments (Tolosa et al. 1995), and were attributed to a different composition of the PCB commercial mixtures used in France and Spain. At the reference stations, mullet were also enriched in tetra- and pentachlorinated congener, and these distributions may well reflect the long-distance transport of less chlorinated congeners and deposition in remote areas, as it is known that atmospheric vapor-particle partitioning may lead to a marine deposition enriched in the more volatile species (Bidleman 1988).

The tissue concentration ranges in Table 2 can be considered representative of pristine, medium and highly contaminated areas, and are consistent with those found in sediments (Tolosa et al. 1995), supporting the close relationship of the red mullet pollutant uptake with concentrations in the benthic environment. The PCB content of red mullet along the Spanish coast was similar to that reported 10 yr earlier for the same fish species (Porte & Albaigés 1993), indicating no apparent decline of this pollutant in the region despite its ban in 1979. In contrast, bivalves, which are

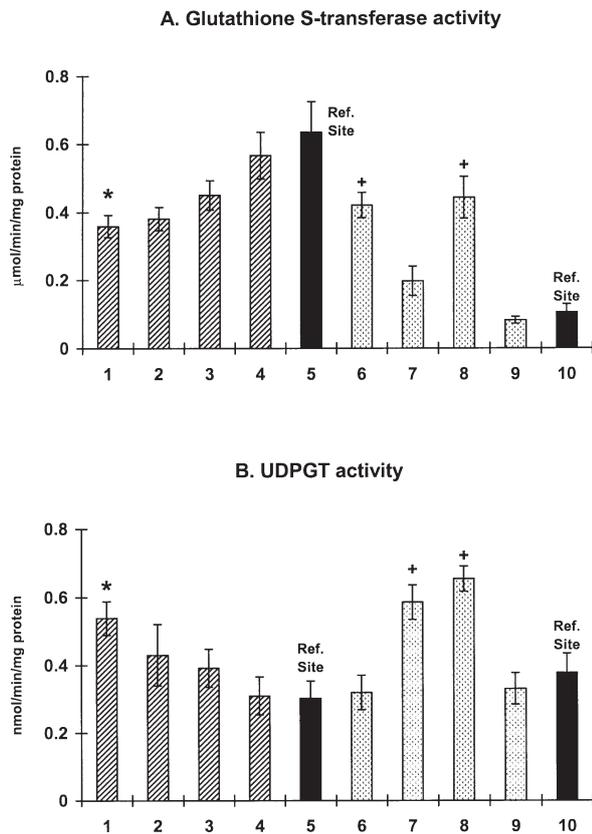


Fig. 7. *Mullus barbatus*. Activities of Phase II enzymes in the liver of red mullet sampled along the NW Mediterranean coast (sites on abscissa, numbered as in Fig. 1). Each value is mean \pm SEM (n = 4 to 6). *Significant difference from reference site at Stn 5; +: significant difference from reference site at Stn 10

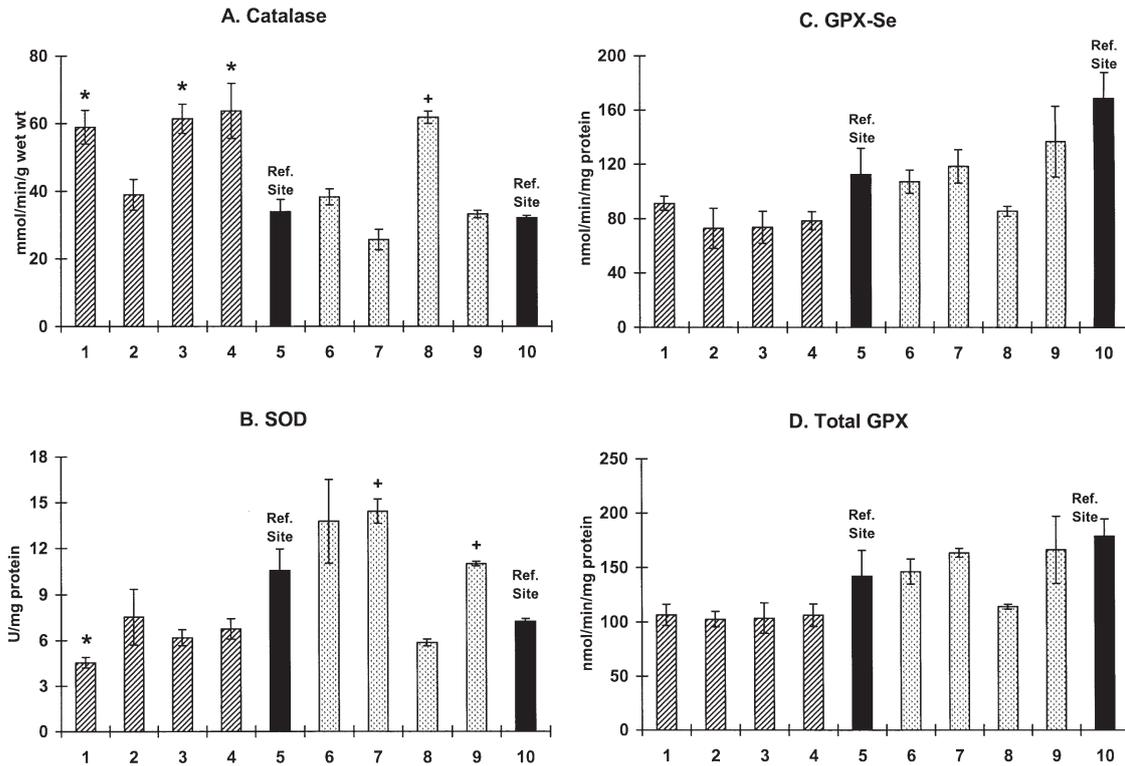


Fig. 8. *Mullus barbatus*. Activities of antioxidant enzymes in the liver of red mullet sampled along the NW Mediterranean coast (sites on abscissa, numbered as in Fig. 1). Each value is mean \pm SEM ($n = 4$ to 6). *Significant difference from reference site at Stn 5; +: significant difference from reference site at Stn 10

primarily associated with the water pollution load, have effectively undergone a significant reduction in PCB content during the same period.

As for PCBs, the levels of total DDTs detected in the Spanish coast are similar to those found 10 yr earlier by Porte (1990). At that time, DDT residues in the muscle tissue of red mullet were in the range of 17 to 172 ng g^{-1} wet wt, depending on their proximity to areas of high DDT concentration. Therefore, there is still no significant decrease in DDT residues in the benthic compartment of the region.

A previous study showed that petrogenic and pyrogenic hydrocarbons were also a major pollution source in coastal sediments (Tolosa et al. 1996). In this respect, the levels of hydroxylated PAHs found in the bile of *Mullus barbatus* at the different stations are consistent with the sedimentary distribution of anthropogenic PAHs, reflecting the fact that red mullet lives in sandy and muddy substrates, where it finds its food, but where it is also exposed to pollutants.

Biomarkers

The hepatic microsomal cytochrome P450 content of *Mullus barbatus* was in the range reported for other

teleosts (0.4 to 0.8 nmol mg^{-1} protein), and was not induced by exposure to pollutants as reported by other authors for other species (Ueng et al. 1992, Haasch et al. 1993), probably due to the fact that our assay measures both constitutive and inducible isoforms of cytochrome P450.

Hence, the inducible forms, CYP1A protein and its catalytic activity (EROD), appear to be sensitive probes for the assessment of pollution exposure in *Mullus barbatus*. A good relation between PCB residues in fish tissue and EROD activity ($r^2 = 0.95$; $p = 0.002$), or CYP1A ($r^2 = 0.76$; $p = 0.010$) was recorded when all samples were pooled, despite the fact that the data derived from 2 different sampling seasons (April and August) and thus included possible seasonal variability (Mathieu et al. 1991). Such a relationship was not observed with bile PAH-metabolites and has been described by other authors (Sleiderink et al. 1995).

Although both CYP1A content and EROD activity showed parallel induction profiles (Figs. 4 & 6), differences among stations were more evident in terms of CYP1A content, viz. EROD activity for Stn 4 was 8.5-fold higher than for the corresponding reference site, whereas CYP1A content was 99-fold higher. Controversial results have been reported from both field and

laboratory studies in this respect. Haasch et al. (1993) found that the largemouth bass *Micropterus salmoides*, exposed to 0.28 mg l⁻¹ β -naphthoflavone, a known CYP1A inducer, showed a 6.4-fold increase in EROD activity 1 d after exposure, and a 25-fold increase in immunoreactive CYP1A. On the other hand, the opposite trend was observed for the rainbow trout *Oncorhynchus mikiss* treated with an i.p. injection of a commercial mixture of PCBs (Clophen A50), Celander & Förlin (1995) reported a 27-fold induction of EROD activity, and only a 4.6-fold induction of CYP1A. However, despite the variability of the responses, the marked geographical differences observed for both EROD and CYP1A and the good correlation with bioaccumulated PCB, provided strong evidence of the usefulness of both measurements as biomarkers of xenobiotic exposure in *Mullus barbatus*.

PROD activity, a measure of the CYP2B subfamily in mammals, was well correlated with EROD activity ($r^2 = 0.81$; $p = 0.016$) in the red mullet, although the activity was very low, 2 orders of magnitude lower than that of EROD. It has been suggested that both PROD and EROD activity are a catalytic measure of CYP1A in fishes (van der Oost et al. 1996). However, in this study we observed a better agreement between CYP1A and EROD ($r^2 = 0.73$; $p = 0.011$) than between CYP1A and PROD ($r^2 = 0.33$; $p = 0.068$).

Cytosolic GSTs were significantly elevated ($p < 0.05$) in fish at polluted stations (6 and 8) during the second cruise in accordance with the role of GSTs in the conjugation of electrophiles produced by P450 monooxygenation, but this was not observed during the first cruise. In contrast, UDPGT was significantly elevated in fish from Stn 1 (first cruise), which was situated close to petrochemical industries and relatively high levels of fluorescent aromatic compounds were present in their bile (Table 2). This was also true for samples from and Stns 7 and 8 (second cruise), which also had the highest CYP1A content, and high concentrations of PCBs in their muscle tissue (Stn 8).

Antioxidant defences consist basically of water-soluble reductants (e.g. glutathione), fat-soluble vitamins (e.g. α -tocopherol), and antioxidant enzymes (Kappus 1986, di Giulio et al. 1989). Antioxidant enzymes were included in this study because of their inducibility under conditions of oxidative stress and their potential role in adaptation to pollutant-induced stress. Catalase activity was significantly elevated in red mullets from the most contaminated Stns (1, 3, 4 and 8). This enzyme catalyses the production of oxygen and water from H₂O₂, and it has been shown in mammalian cells that the enzyme is active at rather high H₂O₂ concentrations (Chance et al. 1979). Thus, catalase normally plays a relatively minor role in the catabolism of H₂O₂ at low rates of H₂O₂ generation (Jones et al. 1981), but

becomes indispensable when the rate of H₂O₂ production is enhanced, as during oxidative stress. This would be in agreement with our findings and those reported by other authors (Rodríguez-Ariza et al. 1993, Bainy et al. 1996, Burgeot et al. 1996). GPX can decompose both H₂O₂ and lipid peroxides, and no significant differences between stations were recorded in this study. Therefore, it is possible that GPX activity would maintain normal cell functions, whereas catalase would form part of a stress-response mechanism (Janssens et al. 2000).

In agreement with previous data by Godin & Garnett (1992) which demonstrated that a low GPX activity could be compensated by high catalase activity, these 2 activities showed a slight negative correlation in the present study ($r^2 = 0.49$; $p = 0.050$). This negative correlation was stronger for total GPX activity, due to some GST isozymes (Se-independent GPX) plus Se-dependent GPX ($r^2 = 0.67$; $p = 0.038$). Total GPX activity did not display significant differences between stations. Generally, the ratio total GPX activity to Se-GPX was rather low (<1.4), and suggests that the major role of GSTs in this fish is the conjugation of xenobiotics rather than an antioxidant function, particularly in low or moderately polluted stations (<1.2: Stns 1, 9 and 10). No clear relationship between the examined pollutants and SOD activity was observed. This enzyme converts superoxide radicals into H₂O₂, and is the primary defence enzyme in combating oxyradicals (Kappus 1985).

Finally, it should be mentioned that not only the analysed pollutants, but also many others, and complex interactions among them and with the biological systems, would be cumulatively responsible for the observed biological responses. Furthermore, factors other than pollutants (namely food availability, reproductive stage, salinity, dissolved oxygen) might be sources of additional stress, and could also influence biochemical markers. Nonetheless, the set of biomarkers used indicated different levels of stress in red mullets paralleling a coastal pollution gradient. It is unclear if this is a sign of impaired health or an adaptational response to a polluted environment. The study provides further support for the use of biomarkers in assessing the health of coastal areas, and also the suitability of *Mullus barbatus* as a sentinel species in the Mediterranean.

Acknowledgements. This work was supported by the POLTOX Project (Generalitat de Catalunya), the Spanish National Plan for Research (PLANYCIT) under project reference AMB95-1092-CE and the UE Project BIOMAR (ENVIRONMENT Programme) under references EV5V-CT94-0550, and ENV4-CT96-0300, and BEEP ref. ENVK3-CT-2000-00025. L.M.G. acknowledges a predoctoral fellowship from CONACYT-Mexico.

LITERATURE CITED

- Albaigés J, Farrán A, Soler M, Gallifa A, Martín P (1987) Accumulation and distribution of biogenic and pollutant hydrocarbons, PCBs and DDT in tissues of western Mediterranean fishes. *Mar Environ Res* 22:1–18
- Bainy ACD, Saito E, Carvalho PSM, Junqueira VBC (1996) Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquat Toxicol* 34:151–162
- Ballschmiter K, Wittlinger R (1991) Interhemisphere exchange of hexachlorocyclohexanes, hexachlorobenzene, polychlorobiphenyls, and 1,1,1-trichloro-bis(p-chlorophenyl)ethane in the lower troposphere. *Environ Sci Technol* 25:1103–1111
- Baumard P, Budzinski H, Garrigues P, Sorbe JC, Burgeot T, Bellocq J (1998) Concentration of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediment and to trophic level. *Mar Pollut Bull* 36:951–960
- Bidleman TF (1988) Atmospheric processes. *Environ Sci Technol* 22:361–368
- Britvic S, Lucic D, Kurelec B (1993) Bile fluorescence and some early biological effects in fish as indicators of pollution by xenobiotics. *Environ Toxicol Chem* 12:765–773
- Burgeot T, Bocquéné G, Porte C, Dimeet J and 5 others (1996) Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. *Mar Ecol Prog Ser* 131:125–141
- Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos* 2:583–588
- Celander M, Förlin L (1995) Decreased responsiveness of the hepatic Cytochrome P4501A1 system in rainbow trout (*Oncorhynchus mykiss*) after prolonged exposure to PCB. *Aquat Toxicol* 33:141–153
- Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59:527–605
- Clarke DJ, George SG, Burchell B (1992) Multiplicity of UDP-glucuronosyltransferases in fish. Purification of UDP-glucuronosyltransferase from the liver of a marine teleost, *Pleuronectes platessa*. *Biochem J* 284:417–423
- Collier TK, Varanasi U (1991) Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in English sole (*Parophrys vetulus*) exposed to environmental contaminants. *Arch Environ Contam Toxicol* 20:462–473
- di Giulio RT, Washburn PC, Wenning RJ, Winston GW, Jewel CS (1989) Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environ Toxicol Chem* 8:1103–1123
- Duinker JC, Schulz SE, Petrick G (1988) Selection of chlorinated biphenyl congener for analysis in environmental samples. *Mar Pollut Bull* 19:19–25
- Escartín E, Porte C (1999) Assessment of PAH pollution in coastal areas from the NW Mediterranean through the analysis of fish bile. *Mar Pollut Bull* 38:1200–1206
- Estabrook RW, Werringloer J (1978) The measurement of difference spectra: application to the cytochromes of microsomes. In: Fleischer S, Packer L (eds) *Methods of enzymology*, Vol 52. Biomembranes. Part C. Biological oxidations, microsomal, cytochrome P-450, and other hemoprotein systems. Academic Press, New York, p 212–220
- Förlin L, Andersson T (1985) Storage conditions of rainbow trout liver cytochrome P450 and conjugating enzymes. *Comp Biochem Physiol B* 80:569–572
- Godin DV, Garnet ME (1992) Species-related variation in tissue antioxidant status. I. Differences in antioxidant enzymes profiles. *Comp Biochem Physiol* 103B:737–742
- Grimalt JO, Gómez-Belinchón JI, Llop R, Albaigés J (1988) Water-phase distribution of hexachlorobenzene in a deltaic environment (Ebro Delta, western Mediterranean). *Chemosphere* 17:1893–1903
- Günzler WA, Flohé L (1985) Glutathione peroxidase. In: Greenwald RA (ed) *Handbook of methods for oxygen radical research*. CRC Press, Boca Raton, FL, p 285–290
- Haasch ML, Prince R, Wejksnora J, Cooper KR, Lech JJ (1993) Caged and wild fish: induction of hepatic cytochrome P-450 (CYP1A1) as an environmental biomonitor. *Environ Toxicol Chem* 12:885–895
- Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. *J Biol Chem* 25:7130–7139
- Janssens B, Childress JJ, Baguet F, Rees JF (2000) Reduced enzymatic antioxidative defense in deep-sea fish. *J Exp Biol* 203:3717–3725
- Jones DP, Eklow L, Thor H, Orrenius S (1981) Metabolism of hydrogen peroxide in isolated hepatocytes: relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H₂O₂. *Arch Biochem Biophys* 210:505–516
- Kappus H (1985) Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. In: Sies H (ed) *Oxidative stress*. Academic Press, London, p 273–310
- Kappus H (1986) Overview of enzyme systems involved in bio-reduction of drugs and in redox cycling. *Biochem Pharmacol* 23:1–6
- Krahn MM, Burows DG, MacLeod WD, Malins DC (1987) Determination of individual metabolites of aromatic compounds in hydrolyzed bile of English sole (*Parophrys vetulus*) from polluted sites in Puget Sound, Washington. *Arch Environ Contam Toxicol* 16:511–522
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 22:680–685
- Lemaire P, Livingstone DR (1993) Pro-oxidant/antioxidant processes and organic xenobiotics interactions in marine organisms, in particular the flounder *Platichthys flesus* and the mussel *Mytilus edulis*. *Trends Comp Biochem Physiol* 1:1119–1150
- Livingstone DR, Archibald S, Chipman JK, Marsh JW (1992) Antioxidant enzymes in liver of dab *Limanda limanda* from the North Sea. *Mar Ecol Prog Ser* 91:97–104
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T, Guengerich FP (1985) Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys* 238:43–48
- Mathieu A, Lemaire P, Carriere S, Drai P, Giudicelli J, Lafaurie M (1991) Seasonal and sex-linked variations in hepatic and extrahepatic biotransformation activities in striped mullet (*Mullus barbatus*). *Ecotoxicol Environ Saf* 22:45–57
- McCord JM, Fridovich I (1969) Superoxide dismutase: an enzymatic function for erythrocyte hemocuprein. *J Biol Chem* 244:6049–6055
- Muir DCG, Norstrom RJ, Simon M (1988) Organochlorine contaminants in arctic marine food chains: accumulation of specific polychlorinated biphenyls and chlordane-related compounds. *Environ Sci Technol* 22:1071–1079
- Pastor D, Boix J, Fernández V, Albaigés J (1996) Bioaccumulation of organochlorinated contaminants in three estuar-

- ine fish species (*Mullus barbatus*, *Mugil cephalus* and *Dicentrarchus labrax*). Mar Pollut Bull 32:257–262
- Porte C (1990) Ecotoxicología de microcontaminantes orgánicos en el medio acuático. PhD thesis, Universidad de Barcelona
- Porte C, Albaigés J (1993) Bioaccumulation patterns of hydrocarbons and polychlorinated biphenyls in bivalves, crustaceans, and fishes. Arch Environ Contam Toxicol 26: 273–281
- Porte C, Solé M, Borghi V, Martínez M and 6 others (2001) Chemical, biochemical and cellular responses in digestive gland of mussels *Mytilus galloprovincialis* from the Spanish Mediterranean coast. Biomarkers 6:335–350
- Rodríguez-Ariza A, Peinado J, Pueyo C, Lopez-Barea J (1993) Biochemical indicators of oxidative stress in fish from polluted littoral areas. Can J Fish Aquat Sci 50:2568–2573
- Roméo M, Mathieu A, Gnassia-Barelli M, Romana A, Lafaurie M (1994) Heavy metal content and biotransformation enzymes in two fish species from the NW Mediterranean. Mar Ecol Prog Ser 107:15–22
- Schlezinger JJ, Stegeman JJ (2001) Induction and suppression of Cytochrome P450 1^a by 3,3',4,4',5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*). Aquat Toxicol 52: 101–115
- Shimakata T, Mihara V, Sato R (1972) Reconstitution of hepatic microsomal stearyl-coenzyme A desaturase system from solubilized components. J Biochem (Tokyo) 72: 1163–1174
- Sleiderink HM, Oostingh I, Goksøyr A, Boon JP (1995) Sensitivity of cytochrome P450 1A induction in dab (*Limanda limanda*) of different age and sex as a biomarker for environmental contaminants in the southern North Sea. Arch Environ Contam Toxicol 28:423–430
- Tolosa I, Bayona JM, Albaigés J (1995) Spatial and temporal distribution, fluxes, and budgets of organochlorinated compounds in NW Mediterranean sediments. Environ Sci Technol 29:2519–2527
- Tolosa I, Bayona JM, Albaigés J (1996) Aliphatic and polycyclic aromatic hydrocarbons and sulfur/oxygen derivatives in NW Mediterranean sediments: spatial and temporal variability, fluxes and budgets. Environ Sci Technol 30:2495–2503
- Ueng TH, Ueng YF, Park SS (1992) Comparative induction of cytochrome P-450-dependent monooxygenases in the livers and gills of tilapia and carp. Aquat Toxicol 23:49–64
- UNEP (United Nations Environmental Programme) (1997) Report of the meeting of experts to review the MEDPOL biomonitoring programme. Athens, Greece, Document UNEP-(OCA)/MED WG. 132/7. UNEP, Athens
- Upshall C, Payne JF, Hellou J (1993) Induction of MFO enzymes and production of bile metabolites in rainbow trout (*Oncorhynchus mykiss*) exposed to waste crankcase oil. Environ Toxicol Chem 12:2105–2112
- van der Oost R, Goksøyr A, Celander M, Heida H, Vermeulen NPE (1996) Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*). II. Biomarkers: pollution-induced biochemical responses. Aquat Toxicol 36:189–222
- Varanasi U, Stein JE, Nishimoto M (1989) Biotransformation and disposition of PAH in fish. In: Varanasi U (ed) Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC Press, Boca Raton, FL, p 93–150
- Walker CH, Livingstone DR (1992) Persistent pollutants in marine ecosystems. SETAC Special Publication Series, Pergamon Press, Oxford
- Yu Y, Wade TL, Fang J, McDonald S, Brooks JM (1995) Gas chromatographic-mass spectrometric analysis of polycyclic aromatic hydrocarbon metabolites in Antarctic fish (*Notothenia gibberifrons*) injected with diesel fuel arctic. Arch Environ Contam Toxicol 29:241–246

Editorial responsibility: Otto Kinne (Editor),
Oldendorf/Luhe, Germany

Submitted: July 9, 2001 Accepted: December 11, 2001
Proofs received from author(s): May 15, 2002