Bioindicators of pollutant exposure in the northwestern Mediterranean Sea

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ABSTRACT: Several bioindicators were used to evaluate the biological and genotoxic effects of marine pollutants near large coastal cities in the northwestern part of the Mediterranean Sea. Three target species of teleosts were selected: red mullet *Mullus barbatus* and 2 types of comber (*Serranus hepatus* and *S. cabrilla*). Induction of ethoxyresorufin-O-deethylase (EROD) activity specific for polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB) was measured in the livers of the fish, and inhibition of acetylcholinesterase (AChE) by organophosphorus insecticides and carbamates was measured in their muscle tissues. Maximal EROD activities (16.8 ± 2.7 to 19.4 ± 4.2 pmol min^{-1} mg protein^{-1}) recorded in red mullet near Barcelona (Spain), Milazzo (Sicily) and Ostia (Italy) indicated exposure to high pollutant concentrations. Inhibitions of AChE activity were low in areas remote from agricultural and industrial activity. The highest inhibitions were measured at sites of heavy industrial and domestic waste, such as Genoa and Naples (Italy), Rio Ter (Spain), Barcelona, and Cortiou (France). Inhibition of AChE activity was higher at a given station for younger individuals 120 to 140 mm in length than for those 160 to 180 mm long. Antioxidant enzyme activities (catalase, superoxide dismutase, glutathione peroxidase and DT-diaphorase) were measured in red mullet livers at 5 stations along the French and Spanish coasts. Catalase activity was highest at Cortiou, consistent with higher levels of pollution, and lower at Mallorca (Balearic Islands). Varying responses were obtained for the other antioxidant enzymes. Glutathione S-transferase (GST), a detoxification enzyme, was also measured in the livers of red mullet fish and found to be significantly higher at Cortiou than at the other locations studied. Chemical measurement of PAH in surface sediment indicated the pyrolytic origin of this contaminant for all stations except Milazzo (petroleum origin). Detection of DNA adducts as a bioindicator of exposure to carcinogenic substances was tested according to 2 complementary assay techniques: enzyme-linked immunosorbent assay (ELISA) and ^{32}P-postlabeling. ELISA revealed maximal quantities of PAH-DNA at Barcelona (15 adducts per 10^8 nucleotides), Cap Finale (Corsica) (20.8) and Milazzo (15.5). The richest adduct profiles were detected by the ^{32}P method at Antibes (France), Santa Ponza (Balearic Islands), Milazzo and Cap Finale, with a maximum of 6.2 adducts per 10^8 nucleotides at Milazzo. This multimarker approach showed that pollutant exposure levels varied according to site. With a sedimentary PAH profile apparently resulting from petroleum pollution, the Milazzo station had the greatest quantity of DNA adducts and the highest inductions of EROD activity and AChE inhibitions in *M. barbatus* and *S. hepatus.*

KEY WORDS: EROD · GST · AChE · Antioxidant enzymes · Biomonitoring · DNA adducts PAH *Mullus barbatus*

INTRODUCTION

Evaluation of the impact of contaminants in coastal areas of the Mediterranean Sea has become a priority for the countries directly concerned. With the support of international organizations (e.g. FAO: Food and Agriculture Organization of the United Nations; UNEP: United Nations Environment Programme; IOC: Intergovernmental Oceanographic Commission) for the coordination of environmental protection, a pilot program for biomonitoring in the northwest Mediterranean (MED-POL) was set up to develop sensitive and

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reliable methods for estimating the state of exposure of organisms to pollutants. Studies of the activity of bio-protection systems in representative marine organisms of coastal benthic ecosystems were performed (GICBEM 1992) along French coasts, and monitoring of biological effects at the Marseille/Fos-sur-Mer pilot site was set up in 1992 in the context of the National Observation Network (NON) (Burgeot et al. 1994a). A biomonitoring program was initiated in the Provence/Alpes/Côte d'Azur region in the northwestern part of the Mediterranean to develop and validate 4 biomonitoring techniques selected in the context of MED-POL, the Mediterranean pollution network (Roméo et al. 1993).

The purpose of the present study was to test the bioindicators recommended by the International Council for the Exploration of the Sea (ICES) and MED-POL for monitoring the marine environment at sites particularly exposed to domestic and industrial waste. Biochemical indicators were measured, along with chemical and genotoxic parameters, in 3 target species of teleosts, red mullet (*Mullus barbatus*) and 2 combers (*Serranus hepatus* and *S. cabrilla*), in coastal areas of the northwest Mediterranean adjacent to large cities. Measurements of hepatic monooxygenase activities and neurospecific activity are now recognized and used as bioindicators of exposure to chemical contaminants (ICES 1994). Ethoxyresorufin-O-deethylase (EROD), specific for polycyclic aromatic hydrocarbons (PAH) and planar polychlorinated biphenyls (PCB), and glutathione S-transferase (GST, EC 2.5.1.18), a detoxification enzyme (Phase II), were measured in the fish livers. Acetylcholinesterase (AChE), specifically inhibited by organophosphorus insecticides and carbamates, was measured in muscle tissues of the 3 target species. The activity of antioxidant hepatic enzymes such as catalase, superoxide dismutase (SOD, EC 1.15.1.1), glutathione peroxidase selenium (GPX, EC 1.11.1.9) and DT-diaphorase (NAD)(P)H-quinone oxidoreductases (NADPH, EC 1.6.99.2) were measured as potential biomarkers of oxidative stress (Di Guilio et al. 1989). Increase in activities of antioxidant enzymes following field (Livingstone et al. 1992) and experimental exposure to xenobiotics (Mather-Mihaich & Di Guilio 1986, 1991a, Markovics et al. 1987) have been detected in a number of fish species, but the responses have not been quantitatively reproducible. The use of these enzymes as biomarkers for environmental oxidative stress is therefore unproven, but merits further research.

The use of xenobiotic DNA adducts as indicators of exposure to genotoxic compounds is a more recent procedure recommended for environmental studies (McCarthy et al. 1989, Varanasi et al. 1989, Stein et al. 1992). In some fish species, the amount of an adduct formed in a target tissue is modulated by Phase II enzymatic reactions, while in other species, the extent of activation of the carcinogen by Phase I enzymes is the major determinant in adduct formation. As a measure of exposure, DNA adducts integrate the factors of uptake, metabolism, and repair and provide an assessment of the dose of carcinogen reaching a target tissue such as liver (Maccubin 1994). In 1964, Dawe et al. suggested the use of bottom-feeding fish to detect environmental carcinogens. We chose to measure DNA adducts in the liver of *Mullus barbatus* because this fish is uniformly distributed along the coasts of the northwestern Mediterranean Sea and because it lives in contact with the sediment. The most widely used method for DNA adducts assessment is the use of radiolabeled chemicals (^{32}P-postlabeling) (Baird 1979). This technique has proven valuable for the chemicals that can be synthesized in radioactive forms with high specific activities (Maccubin 1994). Therefore, an alternative method, such as an immunochemical one, that does not require administration of radiolabeled compounds, is needed. We chose ^{32}P-postlabeling and immunochemical (ELISA) methods based on studies of biomonitoring application in the marine environment (Stein et al. 1993, Ericson et al. 1995) and in humans (Schoket et al. 1993). All of these sensitive methods have advantages and disadvantages. The method chosen for adduct analysis depends on the types of adducts to be detected, the availability of antibodies and the instrumentation that might be especially required for monitoring applications. ^{32}P-postlabeling is a very sensitive but very complex method. Immunochemical detection of BaP DNA adducts is simpler for a routine application; the major adduct formed *in vivo* is the BaP DNA adduct (BPDE) (Sikka et al. 1991).

As the sediment is a possible source of genotoxic chemicals, analyses of PAH in sediment and measurements of DNA adducts (ELISA and ^{32}P-postlabeling) in red mullet livers were performed in the present study to identify the origin of pollution and demonstrate genotoxic alterations of DNA. This study presents the first results obtained on a large geographical scale for the northwestern Mediterranean Sea, enabling biomonitoring of contaminant effects in sensitive areas.

MATERIALS AND METHODS

Fish and sediment

Our cruise followed a route in the form of a loop, from 30 March to 28 April 1994 We first sailed from France toward Spain along the coast, following the dominant northeast current which conveys pollutants toward the southwest. We then passed through the

Balearic Islands of Spain, reached the Sicilian coast, and sailed northward in the Tyrrhenian Sea of Italy, following the Liguro-Provençal current which skirts the continental slope from the Gulf of Genoa to the Catalan Sea (see Fig. 1).

Mullus barbatus, Serranus cabrilla and *S. hepatus* were trawl-fished on the continental shelf to obtain 8 individuals of each species per station. The trawling runs were conducted for 20 min periods. When trawling was impossible, combers were fished with a rod and line. As the cruise covered a considerable geographical area, we were obliged to concentrate our fishing efforts on trawling operations; this explains why sampling was better for the red mullet, which is easier to obtain by this technique. The combers were thus considered as complementary target species.

Individuals of the same size class, without sexual differentiation, were selected to ensure uniform sampling. Mean sizes (in mm) were 129 ± 14.1 to 160 ± 7 for *Mullus barbatus*, 90 ± 12 to 99 ± 14 for *Serranus hepatus* and 131 ± 28 to 186 ± 28 for *S. cabrilla*. Liver and muscle tissues were collected, washed in buffer (KCl 150 mM, Tris 50 mM, pH 7.4) and stored in liquid nitrogen for biochemical analysis. Nineteen stations were sampled in coastal areas near large northwest Mediterranean cities.

Surface sediment collected with a scoop before each trawling run was stored at $-20°C$ for chemical analysis.

Biochemical analyses

EROD measurement. EROD activity was assayed in fish livers using a fluorometric microplate reader, according to the method of Galgani & Payne (1991).

For extract preparation, liver tissue was washed in buffer (Tris 50 mM, KCl 150 mM, pH 7.4) at 4°C and then ground for 10 s in an Elvehjem potter before centrifugation ($9000 \times g$ for 15 min at 4°C). The supernatant was used as an enzymatic solution.

Proteins were measured in supernatant according to the method of Bradford (1976), using BSA as the standard protein. Measurements were performed by microplate spectrophotometer readings at 595 nm, and concentrations were expressed in mg ml^{-1}.

Analysis of EROD activity was performed at 4°C using 10 µl of extract in 200 µl of buffer (Tris 0.1 M, NaCl 0.1 M, pH 8.0) containing 2 µM of 7-ethoxy-resorufin (10 µl) and 0.25 mM of NADPH (10 µl). Activity was determined by kinetic measurement at room temperature in supernatant. The quantity of resorufin formed by catalysis was measured by fluorometry (Galgani & Bocquéné 1989) according to the modified method of Burke & Mayer (1974), with excitation at

544 nm and emission at 584 nm. Kinetics were determined in supernatant at the final 1% dilution. Microplate measurements were performed using a Fluoroscan II fluorometric reader (Grzebyk & Galgani 1991), each plate being read in 1 min. Kinetics were linear for 10 min. An external resorufin standard was used to convert fluorescence units into pmol. Results are expressed in pmol min^{-1} mg protein^{-1}.

GST measurement. Glutathione S-transferase (GST) activity was measured in the cytolic fraction using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, as described by Habig et al. (1974), the final reaction mixture containing 1 mM CDNB and 1 mM reduced glutathione.

AChE measurement. AChE was measured according to the colorimetric method of Ellman et al. (1961) as adapted to a microplate reader. The staining produced by interaction of the DTNB (dithio-bis-nitrobenzoic acid) ion with free thiol groups was measured at 412 nm during acetylthiocholine hydrolysis by AChE.

Muscle tissue from 8 individuals per species obtained at each sampling site was removed and immediately stored in liquid nitrogen. Depending on conditions of local availability, samples of the 3 species were collected as follows: *Mullus barbatus*, 14 stations; *Serranus cabrilla*, 8 stations; and *S. hepatus*, 5 stations.

Specific antioxidant enzymes. Supernatant after $10\,000 \times g$ centrifugation and cytosolic fraction ($100\,000 \times g$ supernatant) were prepared essentially as described in Förlin & Andersson (1985). The tissue was homogenized in a 1:4 tissue weight:buffer volume ratio in 100 mM KH_2PO_4/K_2HPO_4, pH 7.4, containing 0.15 M KCl.

Biochemical measurements were carried out on a UVIKON 941 plus dual-beam spectrophotometer. Antioxidant enzymes were measured essentially as described in Livingstone et al. (1990). Catalase activity was measured in the cytosolic fraction ($100\,000 \times g$ supernatant) using 50 mM H_2O_2 as a substrate. SOD activity was determined by the degree of inhibition of the cytochrome c reduction by superoxide anion radical as described in McCord & Fridovich (1969). The activity of this enzyme is given in SOD units (1 unit = 50% of the inhibition of the cytochrome c reduction). GPX activity was measured by the NADPH consumption during the formation of reduced glutathione by commercial glutathione reductase using 0.8 mM H_2O_2 as a substrate (Greenwald 1985). DT-diaphorase activity was measured in $10\,000 \times g$ supernatant as the dicumarol inhibitable part of NAD(P)H-dichlorophenol (DCPIP) reductase activity. The reduction of DCPIP was measured in the absence and presence of 100 µM dicumarol as described by Benson et al. (1980). Cytosolic and $10\,000 \times g$ supernatant proteins were measured according to the method of Lowry et al. (1951).

Chemical analyses

Chemical measurement of PAHs in sediment.
Extraction and isolation of PAHs: Lyophilization was
performed directly for 48 h (Telstar apparatus) in the
containers used for total surface sediments and without
preliminary freezing From 8 to 15 g of bulk sediment
was extracted by sonication with a dichloromethane
(DCM) methanol mixture (2:1 v/v). The samples were
first Vortex agitated into 75 ml glass centrifuge tubes,
with 50 ml of solvent, then sonicated for 10 min and
finally centrifuged at 5000 rpm for 15 min (Beckman
Model TJ-6 centrifuge); this was all performed 3 times.

Total organic extracts (TOE) were rotary evaporated
under reduced pressure nearly to dryness then dried
under a gentle stream of nitrogen. TOE purification was
performed using 6 ml microcolumn packed with 1 g of
florisil (waters Sep-pack) previously conditioned with
40 ml MeOH, then 20 ml hexane. The PAH containing
fraction was eluted by 20 ml of hexane-DCM (1:1 v/v)
that was concentrated to about 1 ml as described above.
After transferring the mixture into a 1.5 ml pelleter and
drying it under a nitrogen stream, the PAH fraction was
finally rediluted with a solution in iso-octane of Pyrene
d10 (CIL, Woburn, MA, USA) used as an internal stan-
dard. All solvents (Merck, Darmstadt, Germany) were
for organic trace analysis.

GC-MS analysis of PAHs: The PAH fractions were
analysed in a GC 8000 series gas chromatograph cou-
pled with an MD 800 series quadrupole mass spectrom-
eter (Fison Instruments, Milan, Italy). Ion source and in-
terface temperatures were held at 200 and 280°C
respectively, and helium was used as carrier gas at 30 cm
s^{-1}. Injection was performed in the spitless mode, and a
25 m long fused silica column (0.2 mm i.d.) coated with a
0.25 μm thick film of 5% diphenyl methylpolysiloxane
(DB5) was used with the following temperature pro-
gramme: 1 min at 90°C, from 90 to 150°C at 15°C min^{-1}
then from 150 to 310°C at 6°C min^{-1}. The electron energy
was 70 eV and single ion monitoring (SIM) was set as the
detection mode with the following molecular ions as
diagnostic: m/z = 178, 184, 192, 198, 202, 206, 212, 228,
252, 276, 278 (dwell time 70 ms) Peak integration was
performed using the VG-Lab-Base software (Fison
Instruments). The list of PAHs that were systematically
analysed is indicated in Table 2. A standard mixture
containing the 16 EPA (US Environmental Protection
Agency) priority pollutant PAHs was used for identifi-
cation and quantification of PAHs.

Determination of PAH-DNA adducts

Immunochemical method. *Isolation of DNA:* For
DNA isolation, a 10% homogenate was prepared in
Tris 10 mM, pH 7.9, EDTA 1 mM and NaCl 0.4 M, with
sodium dodecyl sulfate added to give a final concen-
tration of 0.2%. Samples were treated with proteinase
K (final concentration 200 μg ml^{-1}) at 37°C for 1 to 2 h,
then extracted with phenol. The nucleic acids were
collected by ethanol precipitation and resuspended in
buffer Hog pancreas alpha-amylase was added to a
final concentration of 200 μg ml^{-1} and samples incu-
bated at 37°C for 1 h. After adjustment to 0.4 M NaCL,
RNase was added to a final concentration of 50 μg ml^{-1}
and samples were incubated for 45 min. Proteinase K
was added to a final concentration of 100 μg ml^{-1} and
incubation continued at 37°C for 45 min. Samples were
extracted with chloroform:isoamyl alcohol, DNA pre-
cipitated with ethanol and resuspended in PBS (phos-
phate buffered saline).

Determination of PAH-DNA adducts by ELISA:
PAH diol-epoxide-DNA adducts were analysed by
competitive ELISA essentially as previously described
(Santella et al. 1992). Briefly, 96-microwell black plates
(Microfluor 'B', Dynatech Laboratories, Chantilly, VA,
USA) were coated with 0.2 ng BPDE-I-DNA (5 adducts
per 10^3 nucleotides). A previously characterized rabbit
antiserum (Poirier et al. 1980) was used at 1:1 600 000
dilution and a standard curve was constructed by
mixing 50 μl diluted antiserum with BPDE-I-DNA
(1.5 adducts per 10^6 nucleotides) in carrier nonmodi-
fied calf thymus DNA (such that 50 μl contained 5 to
150 fmol BPFE-I-deoxyguanosine adduct in 50 μg
DNA) Test samples were assayed at 50 μg $well^{-1}$ after
sonication and denaturation by boiling for 3 min and
cooling on ice. Goat anti-rabbit IgG-alkaline phos-
phatase (Boehringer Mannheim, Indianapolis, IN,
USA) was used at 1:400 dilution and the substrate was
4-methylumbelliferyl phosphate (100 μl, 50 μg ml^{-1}
0.1 M diethanolamine pH 9.6). Fluorescence was read
on a Microfluor reader (Dynatech Laboratories) con-
nected to an IBM computer for data analysis. Samples
were run in triplicate and median values used for
determination of % inhibition. For analytical purposes,
those samples with <15% inhibition were considered
nondetectable and assigned a value of $1/10^8$, an
amount midway between the lowest positive value and
zero. A positive control of DNA isolated from animals
treated with BaP and negative control of human fetal
lung DNA was run with every assay.

Results are expressed as BPDE-I-DNA equivalent
adducts but correspond to the quantification of multi-
ple PAH diol-epoxide-DNA.

[32]P-postlabeling method: DNA was extracted and pu-
rified according to the method described by Reddy &
Randerath (1986) and modified by Pfhol-Leszkowicz et
al. (1991, 1993). Labeled normal nucleotides and residual
ATP were eliminated by an initial chromatography on
polyethyleneimine-cellulose plate in solvent NaH_2PO_4

2.3 M, pH 5.7 overnight. The area deposit containing marked and purified adducts was cut and transferred onto another migration polyethylenimine-cellulose plate, in the solvent: urea 7.65 M format Li 4.77 M, pH 3.5. Two migrations in solvent NaH_2PO_4 0.6 M and urea 5.95 M, pH 6.4 and NaH_2PO_4 1.7 M, pH 6 were conducted perpendicularly to urea 7.65 M format Li 4.77 M, pH 3.5 to attain a better adduct separation.

Statistics

Means ± standard deviations (SD) for EROD activities were calculated from 4 replicates each for 8 fish per station. For AChE, muscle tissue was treated individually and the values given in means ± SD calculated for 8 individuals per station. Measurement of antioxidant enzymes was performed on 6 fish livers and the mean ± SD calculated.

The smallest significant difference test (Z-test) was performed by comparing the station with the lowest induction (the reference station) with the other stations for EROD and stress proteins. For AChE activity, the lowest inhibition was compared with those of the other stations.

Mean DNA adduct measurements by ELISA were calculated from duplicates of a mixture of fish livers from the same station. The method of detecting adducts by ^{32}P-postlabeling as well as the quantification of sediment PAH were qualitative and thus not suitable for statistical analysis.

RESULTS

Measurement of EROD activity

EROD activity was detected in 8 *Mullus barbatus*, *Serranus cabrilla* and *S. hepatus* livers (Burgeot 1994). The red mullet was considered the main target species because of better sampling (14 stations); the 2 combers were used as complementary species (11 stations). Measurement of EROD activity specifically induced by planar PCB and PAH allowed observation of maximal responses for red mullet at the Barcelona (Spain), Milazzo (Sicily) and Ostia (Italy) sites. For the combers, the 3 highest inductions of EROD activity were measured at Milazzo and Civitavecchia (Italy), Cortiou (France) and Santa Manza (France) (Fig. 1).
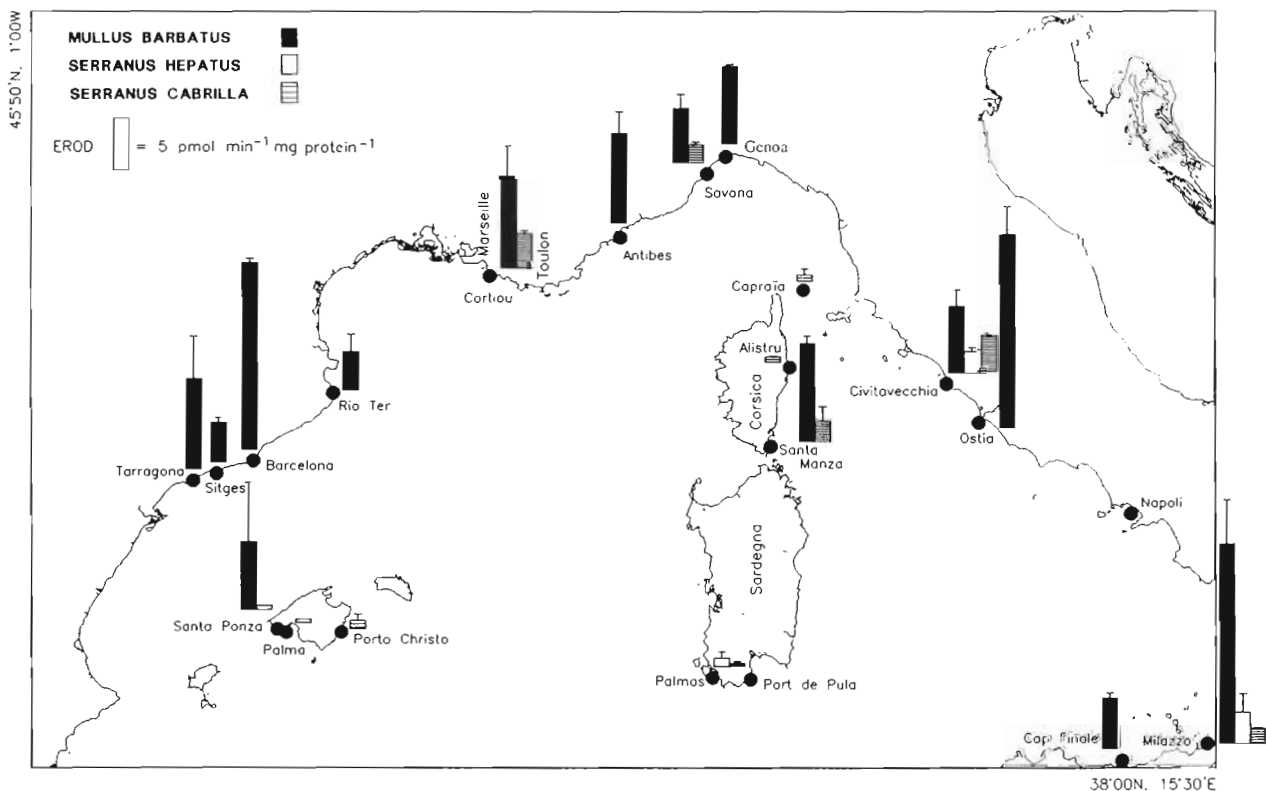

Fig. 1. *Mullus barbatus, Serranus hepatus, S. cabrilla.* Measurement of EROD activity in the livers of 3 target species of teleosts. Mean and SD, calculated from 4 replicates per individual, are shown for each station

Table 1a. *Mullus barbatus*. EROD and AChE activities, DNA adducts (by ELISA and ^{32}P methods), and PAH in sediment at 11 stations. nd: no data; ρ: statistical difference (p < 0.05)

Station	No. of specimens (n)	Body length Mean ± SD (mm)	EROD Mean ± SD (pmol min⁻¹ mg prot⁻¹)	AChE Mean ± SD (U mg prot⁻¹)	Adducts ELISA (PAH equivalent) Adducts/10⁸ nucleotides	Adducts ^{32}P (TOT adducts) Adducts/10⁸ nucleotides	Sediment PAH/TOT sed (ng g⁻¹)	Depth (m)
Cortiou	8	129 ± 1.41	9 ± 2.9 (ρ)	1225 ± 87 (ρ)	7.3	1.2	37507.5	30
Barcelona	8	160 ± 70	18.2 ± 0.1 (ρ)	1564 ± 134 (ρ)	15	1.1	894.39	24
Santa Ponza	8	141 ± 14.1	6.6 ± 5.7	1726 ± 201 (ρ)	8.5	2.9	47.42	58
Cap Finale	8	155 ± 14.1	4.9 ± 0.5 (ρ)	1427 ± 195 (ρ)	20.8	3.5	72.52	20
Milazzo	8	150	19.4 ± 4.2 (ρ)	1645 ± 80 (ρ)	15.5	6.2	183.65	28
Ostia	7	151 ± 42	16.8 ± 2.7 (ρ)	nd	10.8	1.3	nd	nd
Civitavecchia	8	133 ± 7	6.5 ± 1.6 (ρ)	1934 ± 87 (ρ)	nd	1.7	nd	nd
Santa Manza	8	140	9.5 ± 0.7 (ρ)	2250 ± 241 (ρ)	nd	nd	97.45	76
Genoa	8	143 ± 14.1	7.5 ± 0.19 (ρ)	1357 ± 83 (ρ)	nd	nd	19371.8	35
Savona	8	120	5.2 ± 1.4	1698 ± 105 (ρ)	12.8	0.5	3058.46	27
Antibes	8	120	8.6 ± 2.1 (ρ)	3445 ± 213 (ρ)	nd	2	170.89	50

Table 1b. *Serranus cabrilla* and *S. hepatus*. Measurements of EROD and AChE activities at 6 stations. Data represent mean value and SD for EROD and the mean value and standard error of the mean for AChE. Data were compared statistically by a Z-test for the smallest significant difference (p < 0.05). nd: no data; ρ: statistical difference (p < 0.05)

Station	Serranus hepatus No. of specimens (n)	Body length Mean ± SD (mm)	EROD Mean ± SD (pmol min⁻¹ mg prot⁻¹)	AChE Mean ± SD (U mg prot⁻¹)	Serranus cabrilla No. of specimens (n)	Body length (mm)	EROD Mean ± SD (pmol min⁻¹ mg prot⁻¹)	AChE Mean ± SD (U mg prot⁻¹)
Cortiou	nd	nd	nd	nd	8	17.9 ± 14.1	3.4 ± 0.2 (ρ)	2432 ± 181 (ρ)
Santa Ponza	8	90	0.4 ± 0.06 (ρ)	nd	nd	nd	nd	nd
Milazzo	4	90	3 ± 1.8 (ρ)	3220 ± 185 (ρ)	5	172 ± 21.2	1.5 ± 0.06 (ρ)	2932 ± 360 (ρ)
Civitavecchia	8	99 ± 14.1	2.1 ± 0.4 (ρ)	3981 ± 276 (ρ)	5	166 ± 14.1	3.5 ± 0.22	nd
Santa Manza	nd	nd	nd	nd	8	163 ± 14.1	2 ± 1.3	4554 ± 224 (ρ)
Savona	nd	nd	nd	nd	8	186 ± 22	1.7 ± 0.26	3719 ± 263 (ρ)

It was possible to compare the responses of at least 2 different species at 6 stations (Table 1). Milazzo was thus characterized by the highest inductions and Santa Ponza (Balearic Islands) by low inductions for *Mullus barbatus* and *Serranus cabrilla*. The Milazzo site was located next to an oil refinery, the likely source of complex mixtures of specific hydrocarbons. Santa Ponza, situated farther away from urban areas, was characterized by lower inductions of EROD activity.

High inductions were recorded at Civitavecchia for the combers, but at that site the red mullet showed a different sensitivity. Induction for red mullet at Civitavecchia was lower than the highest levels obtained at Ostia and Milazzo (Fig 1). The highest inductions of EROD activity for the combers were always much lower than those for red mullet (Narbonne et al. 1991).

Only 2 stations, Civitavecchia and Milazzo, allowed comparison of the response of all 3 species sampled (Table 1). The Milazzo station was characterized by high inductions for *Mullus barbatus* and *Serranus hepatus* (Fig. 1).

The Cortiou area, situated at a pilot site for the monitoring of biological effects along French coasts (Bur-

geot et al. 1994a), showed high exposure to pollutants, with induction of EROD activity comparable to that of Civitavecchia (Table 1). Measurements of EROD activity recorded at Cortiou in the monitoring context showed that red mullet and comber were exposed to pollution originating from domestic sewage from the city of Marseille, France (Burgeot et al. 1994a, b). The results for EROD activity at Cortiou (9 ± 2.9 pmol min^{-1} mg protein^{-1}) were significantly higher ($p = 0.004$) than those at Rio Ter and Sitges (Spain) (3.7 ± 1.7 and 3.9 ± 0.5 pmol min^{-1} mg protein^{-1} respectively) but only half as high as those for Milazzo, Ostia and Barcelona (Table 1). However, the Santa Manza station, despite its location remote from industrial and domestic sources of waste, had an EROD induction activity for red mullet comparable to that of Cortiou. At the Santa Manza station, high induction levels were also noted in October 1991 for *Mullus barbatus* and *Serranus cabrilla*, but no explanation was found for these high values (Burgeot et al. 1994b).

Comparisons among the different stations showed that observations for the target species did not correlate at the different sites.
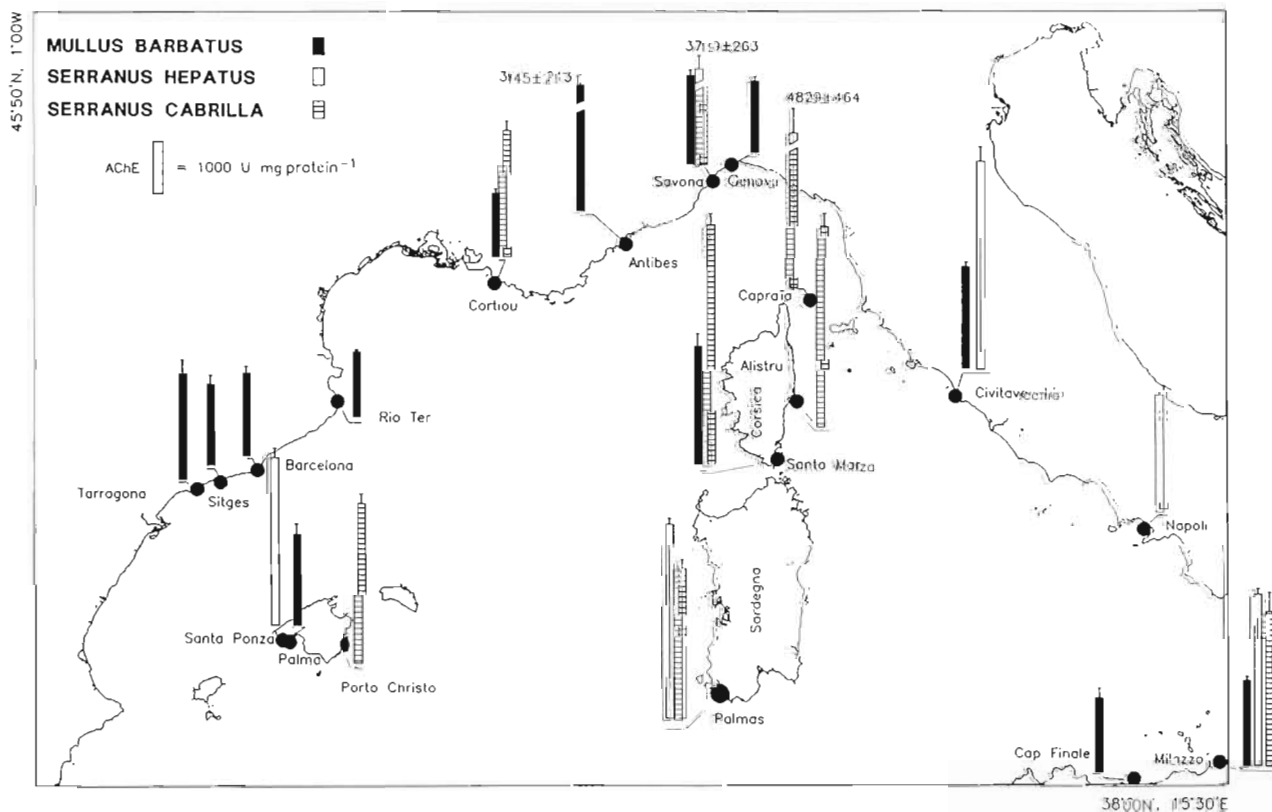

Fig. 2. *Mullus barbatus, Serranus hepatus, S. cabrilla*. AChE inhibition in muscle tissue. Mean and SD were calculated for 4 replicates per individual fished at the same station

Measurement of AChE activity

The abundance of *Mullus barbatus* at 3 stations provided the opportunity to determine AChE activity in muscle tissue as a function of size. Measurements of AChE activity of 3 species at all stations are reported in Fig. 2. AChE activity in *M. barbatus* was found to be inversely proportional to size. Individuals measuring 160 to 180 mm fished at only 3 stations (Milazzo, Civitavecchia and Santa Manza) had very similar activities close to 1500 U mg protein^{-1} (1487 ± 94, 1495 ± 77 and 1450 ± 67 respectively), whereas younger individuals measuring 120 to 140 mm showed higher activities. These data are in agreement with observations concerning other species such as the dragonet *Callionymus lyra* and the comber *Serranus cabrilla* (G. Bocquéné unpubl.). Comparison of AChE activities in individuals measuring 120 to 140 mm showed that the highest values were found at Antibes (France), Santa Manza, Tarragona (Spain) and Civitavecchia; the lowest activities were found at Cortiou, Genoa (Italy), Cap Finale (Sicily), Rio Ter, Sitges and Barcelona (Table 2). The same measurements performed on *S. cabrilla* gave the lowest AChE values at Cortiou (2432 ± 181 U), and the highest at Santa Manza (4554 ± 224) and Capraïa Island (4829 ± 464) northwest of Corsica. AChE activities measured in *S. hepatus* were lowest at Naples, Italy (2217 ± 138) and highest at Civitavecchia (3981 ± 276). These figures, like the others, showed a difference in activity of as much as 50% between extreme values, suggesting the presence of inhibitory substances in the biotope of fish sampled at the stations with the lowest activities.

Measurement of antioxidant and GST enzymes

Some similarities were observable with catalase and GST measurement at 5 stations except for Mallorca (Table 3). Both activities tended to be significantly higher at Cortiou, the station close to the outlet of the Marseille waste treatment facility. Lower activity at Mallorca (reference site) was significant with catalase assays. Intermediate values were detected for catalase activity at the other stations. GPX, however, showed a completely different profile, with the highest activities in the fish sampled at Mallorca and Cortiou. SOD and DT-diaphorase activities were similar at all stations, apart from Sitges and Tarragona, which showed the highest activities, respectively.

Table 2. *Mullus barbatus, Serranus hepatus* and *S. cabrilla*. AChE inhibition values (U mg prot^{-1}) in muscle tissue. Mean and SD were calculated for 4 replicates per individual fished at the same station. 8 individuals were analysed per station; nd: no data

Station	Mullus barbatus	Serranus cabrilla	Serranus hepatus
Alistru	nd	3786 ± 246	nd
Antibes	3445 ± 213	nd	nd
Barcelona	1564 ± 134	nd	nd
Cap Finale	1427 ± 195	nd	nd
Capraïa	4829 ± 464	nd	nd
Civitavecchia	1934 ± 87	nd	3981 ± 276
Cortiou	1225 ± 87	2432 ± 181	nd
Genoa	1357 ± 83	nd	nd
Milazzo	1645 ± 80	2932 ± 360	3247 ± 114
Naples	nd	nd	2217 ± 138
Palma	nd	2851 ± 165	3705 ± 116
Rio Ter	1228 ± 45	nd	nd
Santa Manza	2250 ± 241	4554 ± 224	nd
Santa Ponza	1726 ± 201	nd	3220 ± 185
Savona	1698 ± 105	3719 ± 263	nd
Sitges	1538 ± 169	nd	nd
Tarragona	2012 ± 266	nd	nd

Table 3. Activities of catalase (µmol min^{-1} mg prot^{-1}), glutathione peroxidase (nmol min^{-1} mg prot^{-1}), superoxide dismutase (U min^{-1} mg prot^{-1}), and glutathione S-transferase (nmol min^{-1} mg prot^{-1}) in liver tissue of red mullet. NADPH- and NADH-dependent DT-diaphorase activities (nmol min^{-1} mg prot^{-1}) analysed in supernatant (10 000 × g) of red mullet. Values are mean ±SD (n = 6). *Significant differences, with respect to Mallorca (p < 0.05)

Station	Catalase	SOD	GPX	GST	DT-diaphorase NADH	NADPH
Cortiou	1819 ± 197*	8.1 ± 1.7	111.2 ± 11.5*	3063 ± 143*	2.56 ± 0.56*	1.13 ± 0.19*
Barcelona	1217 ± 206*	8.2 ± 1.6	39.1 ± 2.5*	1262 ± 133	2.68 ± 0.46	1.13 ± 0.28
Sitges	1263 ± 252*	14.4 ± 2.0*	60.8 ± 12.2*	1257 ± 369	3.17 ± 0.33	1.60 ± 0.37
Tarragona	1389 ± 122*	11.0 ± 3.3	47.0 ± 5.6*	1435 ± 131	4.03 ± 0.53	2.18 ± 0.19
Mallorca	704 ± 62	8.2 ± 1.0	147.8 ± 12.6	1603 ± 112	2.65 ± 0.23	1.39 ± 0.12

Measurement of DNA adducts

PAH adducts (ELISA)

Immunochemical determination of PAH-DNA adducts was especially effective for specific contaminations. To test this genotoxic marker for monitoring purposes, measurements were performed in areas subjected to high but not specifically identified anthropogenic inputs. The polyclonal serum used was produced by animals immunized with BPDE-I-DNA. This antibody does not recognize free BDPE molecules. The diol-epoxide guanine N_2 adducts of PAH other than benzo[a]pyrene, such as chrysene and benz[a]anthracene (BaP), possess a stereochemistry close to that of BPDE and are thus capable of being quantified with the BPDE-I-DNA antisera. For this reason, the method allows the assaying of the class of PAH diol-epoxide DNA adducts but data is expressed in BPDE-I-DNA equivalents.

Adducts were measured at only 10 stations because of sampling constraints (Table 1). More than 10 diol-epoxide PAH adducts per 10^8 nucleotides were observed in red mullet DNA at Barcelona, Milazzo, Cap Finale, Ostia and Savona (Italy). In general, higher quantities of adducts were measured at Savona, Barcelona and Milazzo (Table 1), although the greatest amount was assayed at Cap Finale (20.8 adducts per 10^8 nucleotides). EROD activity was highest near the oil refinery at Milazzo, which would appear to be a major source of PAH input (Fig. 1). Measurement of 12.8 adducts per 10^8 nucleotides in red mullet livers at Savona was indicative of PAH exposure.

No correlation was found between PAH adducts and EROD measurements in red mullet, confirming studies in trout (Masfaraud et al. 1992). However, quantities above 10 adducts per 10^8 nucleotides were observed at stations with maximal EROD inductions (Barcelona, Ostia and Milazzo; Table 1). Although there were no direct correlations between adducts and EROD activities, the trend observed would indicate that organisms were exposed to complex mixtures of xenobiotics metabolized by enzymatic catalysis and accumulated in the form of PAH-DNA adducts.

^{32}P-postlabeling PAH adducts

Adduct measurements in red mullet livers by the ^{32}P DNA postlabeling method allowed adduct profiles to be determined nonspecifically (Table 1). The postlabeling method showed the presence of 4 to 9 specific adducts per station and a total of 20 different adduct substances over all individuals (Fig 3). Four to 9 dis-
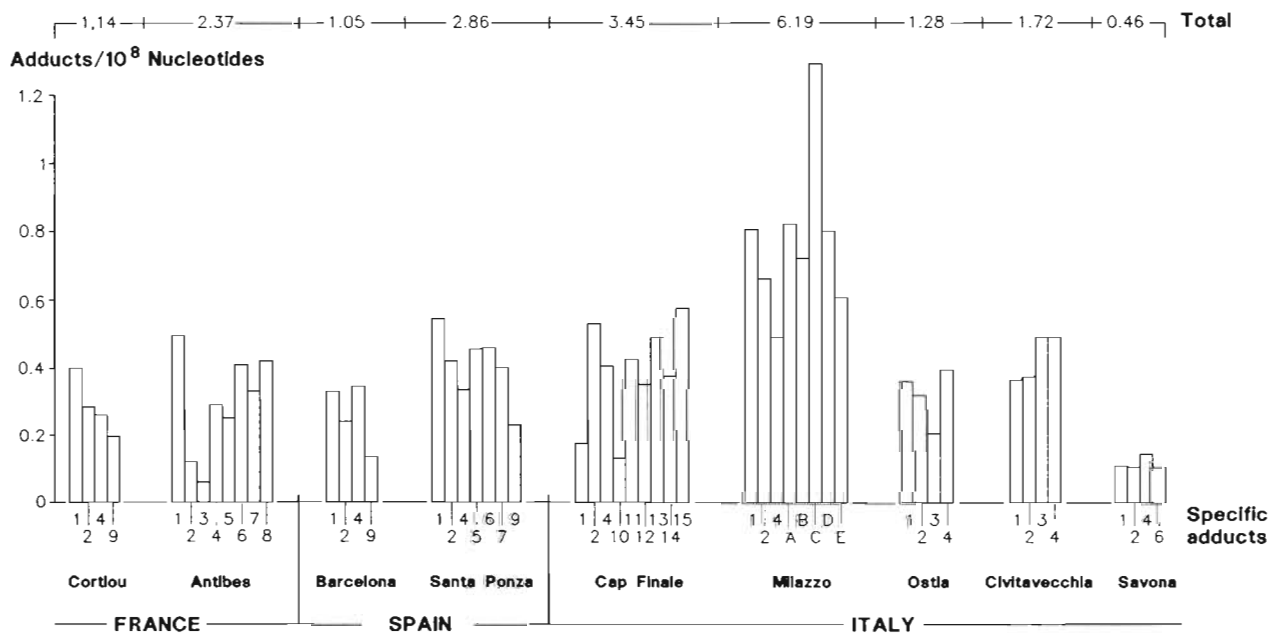

Fig. 3. *Mullus barbatus*. Profile of the different adducts measured by ^{32}P-postlabeling technique in liver tissue at 9 of the 11 stations sampled (see Table 1). Apart from BPDE, designated by the number 1 as guanine N_2 adducts, the other adducts were not identified. Their quantification is arbitrarily designated by numbers ranging from 1 to 15. Adducts designated by letters have not been identified in freshwater fish, whereas numbers designate adducts common to freshwater fish (carp) and seafish. Letter and number designations correspond exclusively to the experiment of the laboratory in which the analysis was performed

tinct adducts were also identified by this analytic method in the mullet *Mugil auratus* from the Adriatic Sea (Kurelec & Gupta 1993) The Cap Finale (9 different adducts), Milazzo (8) and Antibes (8) stations had the richest adduct profiles, while 7 different adducts were identified at Santa Ponza. Similar profiles with 4 adducts were obtained at the Cortiou, Barcelona, Ostia and Civitavecchia stations. No adducts were found at Santa Manza and Genoa. The number of different adducts observed was not systematically indicative of a large quantity of total adducts. Nine different adducts and 3.5 adducts per 10^8 nucleotides were identified at Cap Finale versus 8 and 6.2 adducts per 10^8 nucleotides at Milazzo (Fig. 3). The Cortiou site, though directly influenced by domestic waste input, showed relatively few different adducts.

A very marked difference was noted between the minimum total adducts at Savona (0.5 adducts per 10^8 nucleotides) and the maximum at Milazzo (6.2 adducts per 10^8 nucleotides). EROD activity and adduct measurements (ELISA and ^{32}P), though not correlated, showed a common trend toward high values at Milazzo; a large quantity of PAH adducts was observed by using the 2 measurement techniques at Cap Finale. However, the results obtained at Savona

for the 2 techniques showed different trends. Quantities above 10 adducts per 10^8 nucleotides were noted with the diol-epoxide assay method (ELISA), whereas the lowest total adduct quantity was measured at Savona by the ^{32}P method (Table 1). The BPDE adduct, present in a majority of mammals and the only adduct identified by the postlabeling method in fish, was quantified at all sampling sites by the ^{32}P technique.

Chemical measurements of PAHs in total sediment

Preliminary analytic results of PAHs (Table 4) for a fraction of total sediment enabled us to classify the sampling stations into 3 categories (Fig. 4): those with total PAH of less than 200 ng g^{-1} (Santa Manza, Santa Ponza, Cap Finale, Milazzo and Antibes); those between 894.39 and 3058.46 ng g^{-1} (Barcelona and Savona); and those between 19371 and 37507.5 ng g^{-1} (Genoa & Cortiou).

Surface sediments sampled near large cities at depths of 20 to 76 m allowed identification and quantification of PAH compounds of essentially pyrolytic origin.

Table 4. PAH distribution in total sediment (<2 mm). Concentrations are expressed in ng g^{-1}. Abbreviations for PAHs are explained in Fig. 5 legend

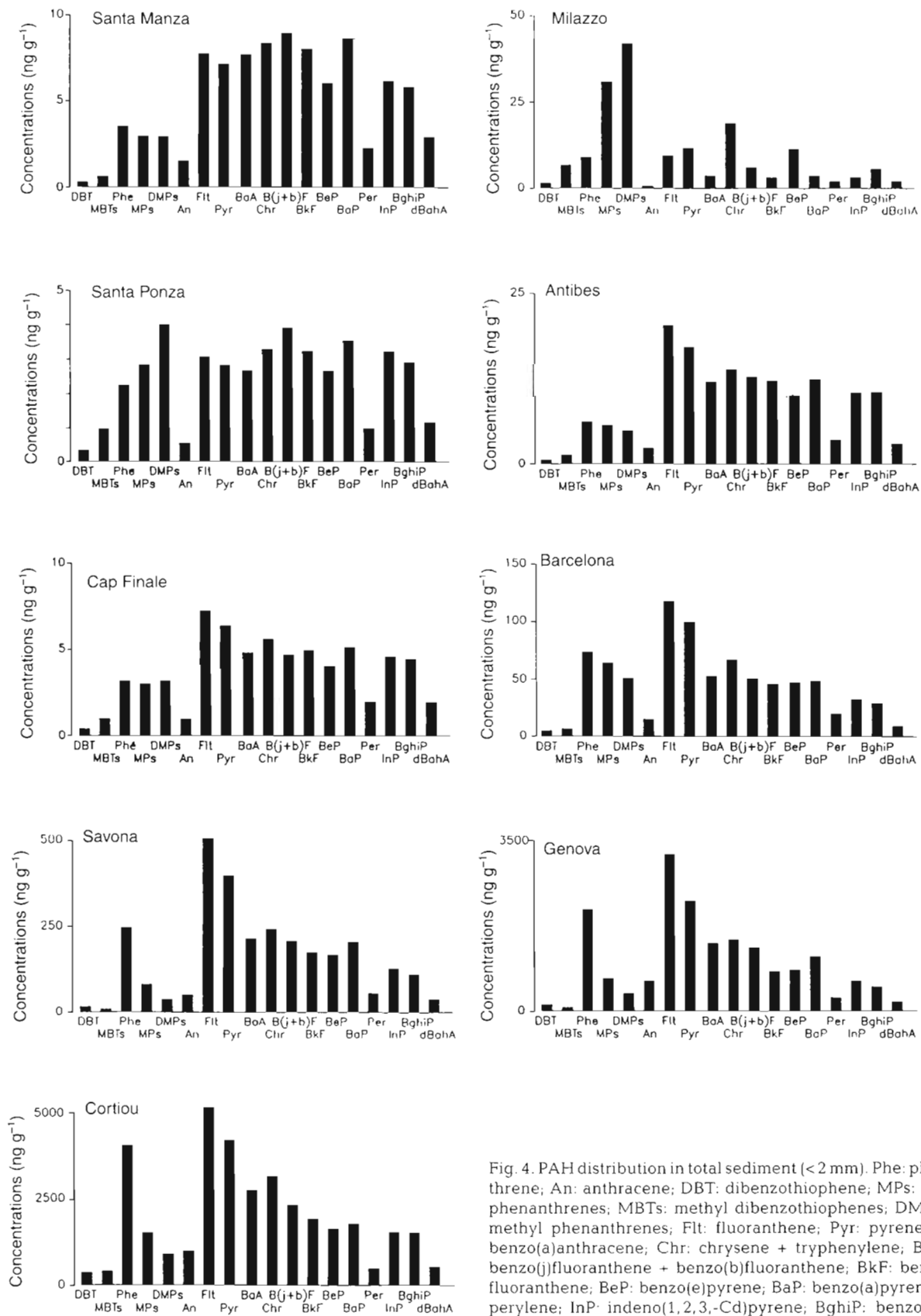| | | | | | Station | | | | |
	Santa Manza	Santa Ponza	Cap Finale	Milazzo	Antibes	Barcelona	Savona	Genoa	Cortiou
DBT	0.290	0.329	0.406	1.48	0.59	5.15	14.78	125.6	352.5
MBTs	0.614	0.967	1.003	6.77	1.32	6.72	8.31	71.9	398.7
Phe	3.555	2.239	3.214	9.04	6.26	74.81	245.53	2127.0	4047.6
MPs	2.958	2.817	3.048	30.90	5.80	65.03	80.92	687.7	1515.7
DMPs	2.939	4.278	3.215	42.65	5.03	51.54	37.02	370.6	906.1
An	1.510	0.539	0.993	0.64	2.36	15.23	51.18	637.0	994.2
Flt	7.726	3.060	7.272	9.62	20.30	119.19	506.67	3271.8	5141.0
Pyr	7.138	2.814	6.421	11.85	17.17	101.21	396.30	2309.0	4194.0
BaA	7.676	2.654	4.845	3.73	12.10	53.50	214.75	1432.9	2758.5
Chr	8.342	3.277	5.628	18.96	13.90	67.98	242.13	1508.0	3153.9
B(j+b)F	8.938	3.910	4.732	6.11	12.81	51.43	209.47	1345.6	2320.7
BkF	7.998	3.223	4.987	3.21	12.27	46.61	175.16	844.9	426.9
BeP	6.013	2.655	4.068	11.54	10.14	47.91	168.94	879.7	1641.3
BaP	8.628	3.541	5.172	3.81	12.53	49.39	205.96	1162.5	1780.9
Per	2.279	0.994	1.982	2.09	3.60	20.29	55.97	283.8	481.8
InP	6.157	3.212	4.619	3.33	10.57	33.30	129.02	634.1	1540.9
BghiP	5.803	2.905	4.486	5.72	10.64	29.75	111.13	517.0	1531.3
dBahA	2.935	1.161	1.968	2.20	3.07	9.24	38.03	197.5	533.7
Total	92	44.6	68.1	174	160	848	2891	18407	35209
MDBTs/DBT	1.13	1.30	2.94	2.47	4.57	2.12	0.57	0.56	2.24
(MPs + DMPs)/Phe	0.60	1.56	3.17	1.95	8.13	1.66	0.50	0.48	1.73
BeP/BaP	0.92	0.97	0.75	0.79	3.03	0.70	0.76	0.82	0.81
Chr/BaA	1.14	1 27	1.23	1.16	5.08	1.09	1.05	1.13	1.15
BghiP/InP	0.99	0.89	0.90	0.97	1.71	0.94	0.82	0 86	1 01
Phe/An	4.1	4.9	4.2	3.2	14.1	2.4	0.3	4.8	2.7

Fig. 4. PAH distribution in total sediment (< 2 mm). Phe: phenanthrene; An: anthracene; DBT: dibenzothiophene; MPs: methyl phenanthrenes; MBTs: methyl dibenzothiophenes; DMPs: dimethyl phenanthrenes; Flt: fluoranthene; Pyr: pyrene; BaA: benzo(a)anthracene; Chr: chrysene + tryphenylene; B(j+b)F: benzo(j)fluoranthene + benzo(b)fluoranthene; BkF: benzo(k)-fluoranthene; BeP: benzo(e)pyrene; BaP: benzo(a)pyrene; Per: perylene; InP: indeno(1,2,3,-Cd)pyrene; BghiP: benzo(g,h,i)-perylene; dBahA: dibenzo-(a,h)anthracene

This contamination is characteristic of the Mediterranean Sea (Raoux 1991, Raoux & Garrigues 1993). Its major components (phenanthrene, fluoranthene and pyrene) are classical representatives of pollution of human origin (Figs. 4 & 5). The lowest values of the structural isomers ratio (Chr/BaA; BeP/BaP; BghiP/Inp

and Phe/An; abbreviations defined in Fig. 4 legend) regularly used to identify petrogenic from pyrolitic origin (Sicre et al. 1987) showed that the sediment was contaminated by compounds with anthropogenic origins at the majority of the sampling stations. The highest values ratio was observed at Milazzo (Fig. 5)

The Milazzo station displayed a characteristic pattern of pollution due to petroleum, which presumably originated from the neighbouring oil refinery. In Milazzo methyl and dimethyl phenanthrene concentrations were more elevated than at the other stations (Table 3).

The ratios between alkylated and parent compounds for phenanthrene (MPs + DMPs)/Phe and dibenzothiophene MDBT/DBT were elevated. They characterized the prevalence of alkylated compounds in the case of petrogenic contamination. The maximum ratio was observed at Milazzo. A peak with a low intensity was also observed at Santa Ponza with an alkylated compounds ratio, though it was not detectable with the other ratios studied (Fig. 5).

DISCUSSION

This scientific cruise was conducted in the vicinity of large and medium-sized cities of the northwestern part of the Mediterranean Sea which are impacted by industrial and domestic pollution. Pollutant evaluation of the marine environment was performed according to a multimarker approach. Three different categories of bioindicators have been developed to diagnose the biological and genotoxic effects of pollutants. EROD has been recommended for national and international biomonitoring programs and AChE and DNA adducts for local studies or those with a particular objective. The third category, antioxidant enzymes, includes biomarkers which require additional research before monitoring applications can be undertaken (ICES 1994).

These different indicators of exposure to xenobiotics were investigated in 3 target species of fish previously studied in the Mediterranean Sea (Mathieu 1990, Narbonne et al. 1991, Sanchez et al. 1993, Burgeot et al. 1994a, b). The results show that these bioindicators gave different res-
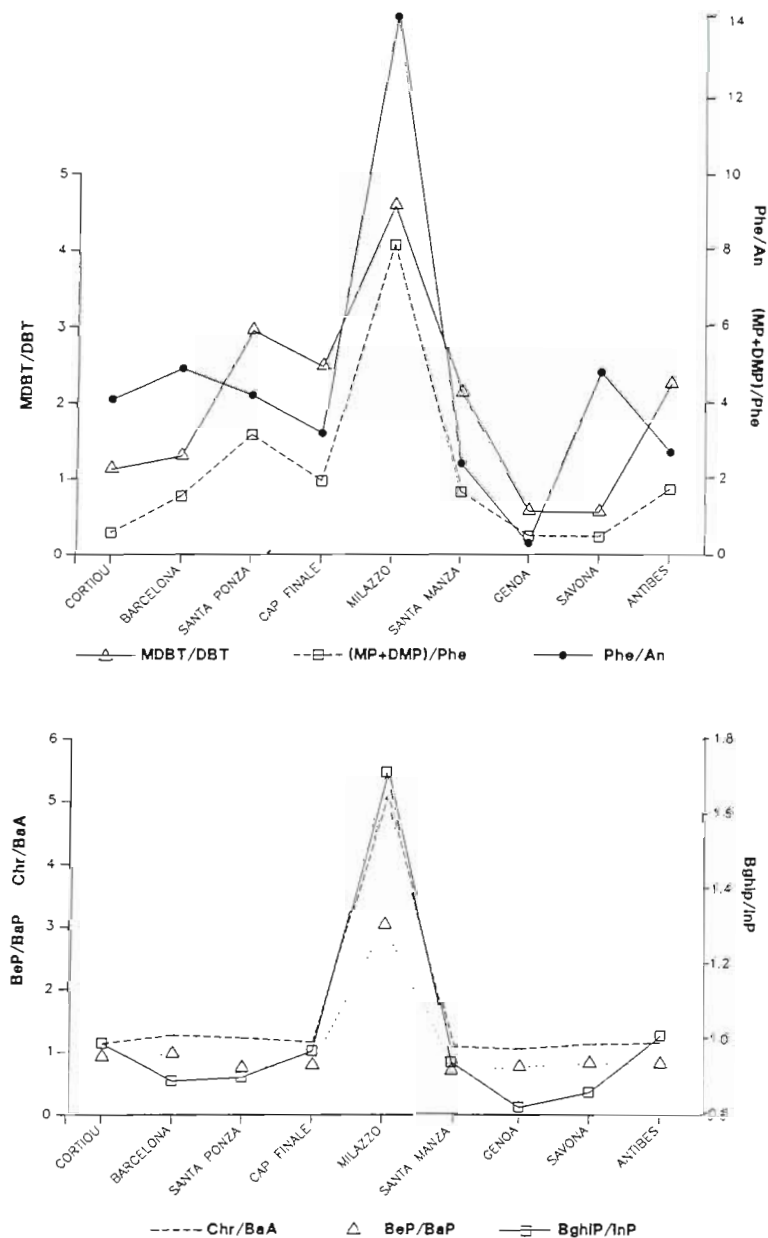

Fig. 5. Ratio of structural PAH isomers. Chemical measurement of PAH in surface sediment indicates the pyrolytic origin of this contaminant for all stations except Milazzo (petroleum origin). MDBT/DBT: methyl dibenzothiophene/dibenzothiophene; (MP+DMP)/Phe: (methyl phenanthrene + dimethyl phenanthrene)/phenanthrene; Phe/An: phenanthrene/anthracene; Chr/BaA: chrysene/benzo(a)anthracene, BeP/BaP: benzo(e)pyrene/benzo(a)pyrene; BghiP/InP: benzo(g,h,i)perylene/indeno(1,2,3,-Cd)pyrene

ponses according to site. EROD and AChE measurements allowed us to obtain information about the exposure state for each fish species (Figs. 1 & 2). Observations obtained by the simultaneous use of these tests provided more consistent data, allowing a first evaluation to be made.

EROD

The EROD study focused on the red mullet *Mullus barbatus* because hepatic capacities of this species are better expressed by EROD variation than are those of the combers *Serranus hepatus* and *S. cabrilla* (Funari et al. 1987, Mathieu 1990). The highest inductions of EROD activity for combers were much lower than those for red mullets (Narbonne et al. 1991). The red mullet can also be trawl-fished, allowing more rapid and extensive sampling (Burgeot et al. 1994b). Inductions of EROD activity in red mullet at the Barcelona, Ostia and Milazzo stations were 2 to 3 times higher than those at other stations, indicating the presence of significant contaminant concentrations. Inductions of EROD activity at the Cortiou and Santa Manza stations were slightly lower but significant (Table 1). The distance from the source of pollution can influence the exposure level of fish, but it is very difficult to interpret this phenomenon on a large geographical scale. The geographical situation of the station is primarily set by trawl-fishing feasability and characteristics of the ocean bottom. In order to more precisely interpret EROD variation in different areas in the future, this factor of variation must be more closely considered.

The 2 comber species, which have a different way of life than that of the red mullet, showed particularly high inductions at the Cortiou, Civitavecchia and Milazzo stations. Red mullet is more sensitive than comber to EROD activity, and induction is rapid. However, the activity is subject to seasonal variations (Mathieu 1990). EROD activity in males, in particular, is intensely expressed in May during the breeding period. During our study in April, we observed an increase in gonad maturation between the Cortiou station (sampled 10 d earlier) and the Cap Finale station, in conjunction with a 4°C increase in water temperature. As the sexual maturation factor influencing EROD activity can hinder interpretation of results, a multimarker approach is desirable to compensate for this type of problem. PAH measurements in sediment and DNA adduct measurements in liver tissue can provide complementary data on the origins and genotoxic impact of PAH. The identification of individual PAH in sediment can facilitate the determination of specific compounds responsible for biological effects.

AChE

Organophosphorus insecticides and carbamates are currently considered to be the most powerful cholinesterase inhibitors. Most of these substances induce significant inhibitions at concentrations in water of between 0.1 and 1 ng l^{-1}. The presence of these substances in Cortiou station sediments was probably due to the proximity of the outlet for waste waters from the Marseille urban area (with 3.5 million inhabitants), although it is difficult to be certain in the absence of precise chemical data.

In general, the highest values of AChE were measured in areas free of notable industrial or agricultural contamination (south Corsica, Capraïa Island), whereas the lowest values were observed in highly contaminated sites (Gulf of Genoa, Cortiou, Naples, Barcelona). Data are scarce on the concentrations of organophosphorus and carbamate compounds in the different marine compartments. However, organophosphorus levels of up to 300 ng g^{-1} in fish tissue have been measured in the Ebro delta south of Barcelona (Barceló et al. 1990, UNEP/WHO 1991). Traces of ethylparathion and malathion have been detected in Camargue drainage water in France. Analytic problems make it difficult to obtain data on the presence of these substances in the marine environment because each substance must be identified individually. About 40 different organophosphorus compounds and about 20 distinct carbamates were used in France in 1993. Evaluation of organophosphorus and carbamate concentrations is thus extremely difficult, and the risk of underestimation is great. Moreover, a recent study (Bocquéné et al. 1995) confirmed the highly synergic effects of associated organophosphorus and carbamates. All this information on the sensitivity and specificity of the cholinesterase target and the synergy of inhibitory effects makes it likely, in the absence of chemical data, that there are inhibitory effects related to organophosphorus and carbamates in coastal marine sediment.

Antioxidant and GST enzyme measurement

Induction of antioxidant and GST enzymes were measured in *Mullus barbatus* liver tissue at 5 stations along the French and Spanish coasts to demonstrate alterations due to the presence of free radicals from pollutant metabolism (Table 3). Although relations between deaths or the existence of neoplasms and alterations in oxidative stress have not yet been clearly elucidated, inductions of antioxidant enzyme activities during exposure to PAHs and PCBs or even fungicide xenobiotics have been observed in flatfish (Chipman et

al. 1992). However, overall responses are quite variable (Livingstone et al. 1992).

Catalase and GST were highest at the most contaminated area, which appeared to be a pollutant-related response, particularly for catalase. In contrast, GPX showed elevated activities at Cortiou (highly polluted) and surprisingly, at Mallorca (clean sea), indicating that factors other than pollution may influence this enzyme. Similar varying results were described by Mather-Mihaich & DiGuilio (1991b), who reported a dose-related increase in catalase activity with no change in SOD or GPX in liver tissue of the catfish *Ictalurus punctatus* exposed to bleached kraft mill effluent.

No elevation of DT-diaphorases or SOD activity was evident at Cortiou. However, DT-diaphorase, NADH- and NADPH-dependent, were maximal at Tarrgona and in areas where oil-related activities occur. In mammals, this enzyme appeared to be linked with cytochrome P450 IA1, but very little is known about this in fish. Moreover, the assay conditions may not be the best ones to measure its activity. The degree of inhibition by dicumarol of NADPH- or NADH-dependent DCPIP reductase activity in *Mullus barbatus* is generaly low and variable (0.8 to 16%) as reported by Livingstone et al. (1990) for *Limanda limanda*.

From these results, we conclude that the use of antioxidant enzymes alone as biomarkers of oxidative stress is far from sufficient (Peters et al. 1994). However, an integrated approach with sets of different biomarkers of exposure (e.g. EROD) or damage (e.g. DNA adducts), plus detailed chemical characterization of the sampling sites, would contribute to an evaluation of the biological effects of pollutants in the marine environment.

PAH in sediment

PAH measurements in sediment allowed us to define the origins of specific pollutants at each site. Two sources of oceanic PAH pollution are classically noted: natural continental sources and anthropogenic sources. The profiles obtained in the vicinity of large cities are characteristic of compounds of anthropogenic origin. In our study, the PAH contamination patterns in surface sediments were generally very similar, except at the Milazzo station, which had high levels of methyl and dimethyl phenanthrene (Fig. 4). These 2 compounds are fairly representative of petroleum-induced pollution, which appeared due to the presence of an oil refinery in that area. At the other stations, the major role of phenanthrene, fluoranthene and pyrene compounds was indicative of the pyrolytic contamination characteristic of areas of industrial and urban activity.

The expression of total PAH showed 3 contamination trends. Maximum contamination was registered at Cortiou (Table 4), a station situated several meters from the outlet for urban waste from Marseille. Concentrations for parent PAH currently analyzed were in the same range at the Barcelona, Savona, Genoa and Cortiou stations as those in the Ebro delta in Spain (Albaigés et al. 1982) or in regions along the Spanish coast situated near urban areas (Albaigés et al. 1984). However, results at the Cortiou station were far higher than those measured between Marseille and the Rhône delta in southern France (Mille et al. 1981, 1982) or even those obtained within the Rhône delta (230 to 1720 ng g^{-1}) (Milano et al. 1986). Nonetheless, interpretation of the distribution pattern for total PAH, considered as evidence for the identification of pollutant origin, is still difficult. All these results indicate the need for a more thorough study of the environmental geochemistry and specific activities at each site. However, these results show that the stations studied are subjected mainly to contamination of pyrolitic origin, except for Milazzo, which is subjected to characteristic inputs of petroleum products.

DNA adducts

Measurements of DNA adducts by ^{32}P-postlabeling enabled us to determine the presence of adducts without identifying them singularly (except BPDE-I-DNA) (Fig. 3). PAH diol epoxide-DNA adducts were the only adducts which could be identified by the immunochemical method.

The profiles of DNA adducts obtained allowed for differentiation of specific contamination features among sites probably subjected to human pollution sources, but also to different natural variables such as temperature, water depth and environmental oxygen pressure which can influence PAH transformation in the marine context. Assays of BPDE adducts by the ^{32}P method showed that BPDE was present at all stations, but at varying concentrations (Fig. 3). Quantification of BPDE-I-DNA equivalents by ELISA allowed us to observe wide variations between the different sites (Table 1).

The complementary data obtained by the 2 methods are useful for evaluating the genotoxic effect of PAH. PAH-DNA measurements, currently used in human monitoring as an indicator of pollution (Poirier et al. 1980), have been compared with the ^{32}P-postlabeling method performed on humans (Schoket et al. 1993). Like our results for fish, this comparison exercise on humans allowed us to observe the lowest values with the ^{32}P-postlabeling method in spite of a larger measurement adduct spectrum than that of the ELISA method. The discordance observed between the

2 methods could be due to the DNA extraction method used. An insufficient selective DNA extraction with the ELISA method could induce the detection of RNA adducts and an overevaluation of DNA adducts. A cross reaction with PAHs possessing the same stereochemistry as BaP could be quantified by ELISA and not by ^{32}P-postlabeling because of their small size. Lastly, loss of adducts could be improved during the enrichment phase with P1 nuclease. P1 nuclease could involve an 80% loss of adducts on the C_8 of the guanine (A. Pfhol-Leszkowicz unpubl.).

However, further studies using the 2 measurement methods (^{32}P-postlabeling and the immunochemical assay) in relation with PAH chemical measurements in sediment should be performed to facilitate the interpretation of results, a process which is often difficult and related to seasonal variations (Kurelec & Gupta 1993). The use of markers such as BPDE-I-DNA equivalents indicates that the exposure of fish to complex PAH mixtures results from superimpositions of emissions from several sources (Lipiatou 1989). For example, large quantities of PAH-DNA adducts were recorded at the Milazzo site with both methods. The high EROD activity measured at this same site suggests that fish living there are exposed to heavy pollution and to bioactive chemical substances. The Cap Finale site, which had a very rich DNA adduct profile with the ^{32}P method, also showed high amounts of PAH-DNA with the ELISA method. Although PAHs represent only a small fraction of the complex mixtures of petroleum hydrocarbons in the environment, these results suggest that adduct measurements are more particularly effective at sites highly contaminated by PAH (Dunn et al. 1987, Stein et al. 1992). These studies confirm other *in vivo* experiments (Maccubin 1994) which support the contention that the sediment is a possible source for at least some of the genotoxic chemicals to which the fish are exposed. But the ultimate use of the 2 DNA adduct assay methods for monitoring purposes require further studies to evaluate their interest for the monitoring of the marine environment.

In the perspective of genotoxic studies, the monitoring of DNA adducts in fish livers could provide an interesting tool for observing genetic alterations related to long-term exposure. The poor capacities for repair of DNA alterations in fish (Dunn et al. 1987) indicate that data are required concerning the different exposure possibilities. The formation of DNA adducts is a key phase in the onset of cancer, providing a link between genetic mutations in fish livers and the genotoxic effect of chemical substances in the marine environment. Even though adducts have been included among the useful exposure biomarkers for the monitoring of the aquatic environment, certain factors are capable of introducing confusion. The for-

mation of adducts in unpolluted areas, seasonal variations in their formation (Kurelec & Gupta 1993) and the mechanisms of DNA repair dependent on exposure time need to be considered before any attempts are made to interpret carcinogenic effects in environmental monitoring. Another difficulty in field study is the route and the duration of exposure of wild fish to a complex mixture of pollutants. In spite of this difficulty, the concordance of data observed at the Milazzo site, which showed very strong enzymatic EROD activity as well as a high amount of DNA adducts in *Mullus barbatus* liver tissue, indicates the sensitivity of these biomarkers to the sediment contaminated by petroleum products.

In conclusion, the results obtained here suggest that the complementary use of several indicators of biological and genotoxic pollution facilitates evaluation of the state of exposure of these organisms to complex mixtures of chemical pollutants. *Mullus barbatus* were proven suitable for bioindicator analysis. Exposure to insecticides or hydrocarbons were identifiable at the most contaminated sites by using enzymatic bioindicators (EROD and AChE). The identification of individual PAHs in sediment can facilitate the determination of specific compounds responsible for biological effects. For this initial field study, the choice of areas in the northwestern Mediterranean Sea near large cities provided a preliminary estimation of genotoxic alterations revealed by DNA adduct assays. The application of parameters recommended by the international community (ICES and MED-POL) to sensitive sites, the use of a multimarker approach and the assaying of PAH in sediment provided experience and data which will be beneficial to the future use of certain bioindicators for long-term monitoring.

With respect to field studies, more work is needed in identifying the adducts observed in wild fish, especially for the complex pattern observed by using ^{32}P-postlabeling In order to complete the multimarkers approach, it would be interesting to estimate tumors and neoplasia in wild fish combined with the evaluation of critical genetic targets of DNA adducts such as tumors, suppressor genes and oncogenes.

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