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Chemical, biochemical and cellular responses in the digestive gland of the mussel *Mytilus galloprovincialis* from the Spanish Mediterranean coast

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Mussels (*Mytilus galloprovincialis*) were sampled in March 1996 from five stations along the Western Mediterranean coast (Barcelona, Ebro Delta, Alboraya, Cullera, Denia) corresponding to urban, industrial and agricultural areas. Different biochemical and cellular markers were determined in the mussels in order to assess the effects and/or exposure to pollutants. The cytochrome P450 system, acetylcholinesterase and metallothioneins were among the biochemical markers selected for the study. Histochemical analysis of ß-glucuronidase and catalase activity were performed as marker enzymes for lysosomes and peroxisomes. Chemical analyses indicated that mussels from Barcelona and Denia as highly exposed to polycyclic aromatic hydrocarbons (PAHs) (1.8–2.7 $\mu g\,g^{-1}$ w.w. against 0.02–0.10 $\mu g\,g^{-1}$ w.w.), and polychlorinated biphenyls (PCBs) (132–260 $ng\,g^{-1}$ w.w. against 8–24 $ng\,g^{-1}$ w.w.). This was in agreement with changes in lysosome structure and higher number of peroxisomes in those organisms. High levels of metals (particularly Cr and Cu) were recorded in the digestive gland of Alboraya mussels, which also had elevated metallothionein content (28 $nmol\,g^{-1}$ w.w.) in comparison with the other stations (15–20 $nmol\,g^{-1}$ w.w.). Benzo(*a*)pyrene hydroxylase (BPH) activity indicated Cullera and Barcelona as possibly polluted sites. The results support the usefulness of the biomarker approach to assess and diagnose environmental pollution. The use of a battery of biomarkers at different levels of biological organization coupled with chemical analysis is highlighted.

Keywords: biomarkers, metallothioneins, cytochrome P450, acetylcholinesterase, lysosomal enlargement, peroxisome proliferation, mussels, pollution.

Introduction

Mussels and other bivalves are widely used as sentinel organisms in pollution-monitoring programmes (Goldberg *et al.* 1978, O'Connor 1996). Their sessil and filtering habits and ability to bioaccumulate most pollutants make them suitable organisms to assess the concentration of selected chemicals in their surrounding environment. Pollution assessment using mussels as sentinel organisms along the Spanish Mediterranean coast started in the early 1980s (Risebrough *et al.* 1983); since then, a number of contaminants have been regularly monitored. Most of the data available refer to tissue levels of metals, polycyclic aromatic hydrocarbons

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(PAHs), organophosphorous pesticides and organochlorinated compounds (Porte and Albaigés 1993, Pastor *et al*. 1994, Solé *et al*. 1994). However, information on the fate and toxicity of these and other chemicals that are currently released into the marine environment is scarce.

Thus, techniques for measuring biological effects of contaminants are being incorporated into 'mussel watch' programmes with the aim of integrating the effects of all those contaminants, and providing information on their significance for the organism health (Krishnakumar *et al*. 1994, Livingstone *et al*. 1995, Porte *et al*. 2001). Among those biological techniques, biochemical and cellular responses have been the subject of much research and have been used as early warning signals of exposure and/or effect of pollutants (McCarthy and Shugart 1990). In this context, the present study was designed to integrate chemical analysis of selected contaminants in mussel tissue (metals, PAHs, PCBs, DDTs) with several biochemical (metallothionein content, cytochrome P450 system, acetylcholinesterase inhibition), and cellular responses (lysosome enlargement and peroxisome proliferation), with the aim of demonstrating linkages between them.

Metallothioneins (MTs) were determined in gills and digestive gland and used as a tool to monitor environmental impact of metals (George and Olsson 1994). Induction of the cytochrome P450 monooxygenase system was applied to diagnose exposure to certain organic pollutants; although the nature of the response of this system in molluscs is still under discussion (Peters *et al*. 1999). Acetylcholinesterase activity was determined in gills and intended to diagnose organophosphorus pesticide (OPs) exposure. Some areas of the Mediterranean coast are affected locally by OPs, but due to their relatively fast biodegradation tissue residues are very seldom detected in biota (Escartín and Porte 1997).

Besides biochemical responses, two cellular markers, lysosomal enlargement and peroxisome proliferation, were selected for the study. Digestive cells of bivalves contain a very well-developed endolysosomal system, which is involved in the uptake and digestion of food material and the accumulation of metallic and organic contaminants. The ability of molluscan digestive cells to accumulate metallic contaminants in lysosomes has been used as a marker of metal bioavailability (Soto and Marigómez 1997). Contaminants are reported to cause a significant increase in lysosomal size, accompanied in certain cases by increases in organelle numbers; and this has been successfully used as a general marker of pollutant impact (Moore *et al*. 1987, Etxeberria *et al*. 1995, Marigómez *et al*. 1996). Peroxisome proliferation is another major effect of xenobiotics, such as phthalate ester, plasticizers, PAHs, etc. (Beier and Fahimi 1991, Cajaraville *et al*. 1997, Krishnakumar *et al*. 1997, Cancio *et al*. 1998). Field studies have indicated significant correlation between PAH and PCB residues in mussels and peroxisome proliferation in digestive tubules (Krishnakumar *et al*. 1995).

Hence, in order to undertake the integrated monitoring strategy, mussels (*Mytilus galloprovincialis*) were collected from five sites along the Spanish Mediterranean coast (figure 1), including: (1) two urban and industrial areas, Barcelona and Alboraya—in the metropolitan area of Valencia—(2) Denia, a tourist resort surrounded by orange tree fields, and with intensive maritime traffic and plastic industries, (3) Cullera, a recreational area with intensive agriculture (vegetable crops and orange trees), and (4) the Ebro Delta, a Natural Park, where bivalve farms are located.

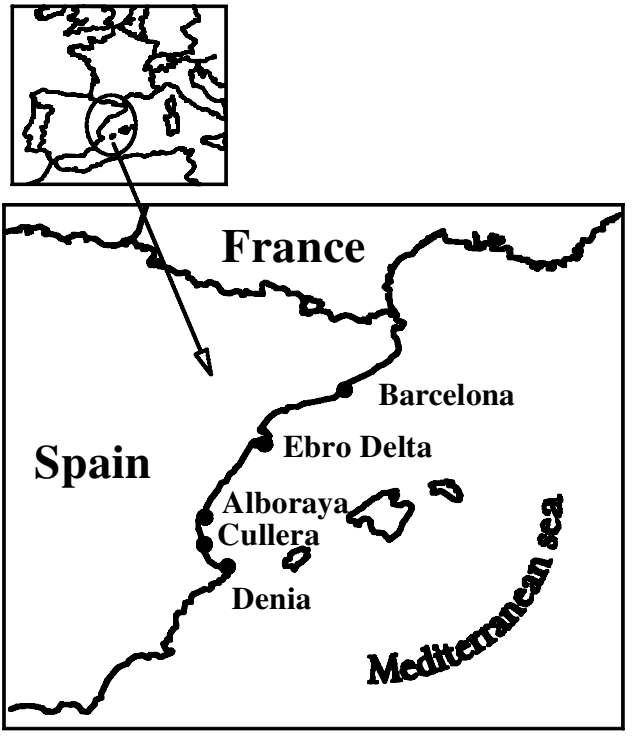

Figure 1. Map of the area of study

Materials and methods

Sample collection and preparation

Mussels (*Mytilus galloprovincialis*, 55–70 mm in length) were collected in March 1996 from five sites along the Western Mediterranean coast in Spain (figure 1). Digestive gland and gills were immediately dissected, frozen in liquid nitrogen and stored at −80°C until analysis.

Chemical analysis

Analysis of organic contaminants. About 2 g pooled digestive glands was used for single chemical analyses. Digestive glands were cut into small pieces, saponified with aqueous 6 N NaOH and extracted with 3 × 30 ml hexane:dichloromethane (4:1) to obtain the total organic extract, which was further cleaned up by liquid chromatography (5% water deactivated silica-alumina column) as described (Aceves *et al.* 1988). Mono- and dicyclic aromatic hydrocarbons were eluted with 30 ml hexane:dichloromethane (9:1), and PAHs with 20 ml hexane:dichloromethane (8:2).

PCBs and DDTs were collected in the first fraction I. DDTs were mainly in the DDE form due to saponification of the homogenates. This fraction was injected into a gas chromatograph coupled with an electron-capture detector (GC-ECD) at 300°C (Hewlett Packard 5890). The column, a 50 m × 0.25 mm i.d. CP-Sil 8 CB fused silica (Chrompack, Middelburg, NL, USA), was programmed from 80 to 180°C at 15°C min^{-1} and from 180 to 280°C at 3°C min^{-1}, keeping the final temperature for 15 min. The carrier gas was helium at a linear flow-rate of 50 cm s^{-1}. The injector temperature was 280°C. Quantitation was performed using an external standard calibration mixture of selected PCB congeners (IUPAC nos 28, 52, 101, 118, 153, 138, 180) supplied by Promochem (Wesel, Germany). These congeners were quantified separately and the PCB concentrations defined as its sum. *p,p′*DDE and *p,p*'DDT were also determined and quantified.

Individual determination of PAHs was achieved by gas chromatography coupled to mass spectrometry in the electron-impact mode (GC-MS-EI) with selected ion monitoring (SIM), using a Fisons GC 8000 series chromatograph interfaced to a Fisons MD800 mass spectrometer. Characteristics of the column and operating conditions were those described in Solé *et al.* (1996). Individual PAHs were identified and quantified by comparison of retention times of reference compounds. A standard mixture

containing 14 individual PAHs, namely naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, indeno-(1,2,3-*cd*) pyrene, dibenz(*ah*)anthracene and benzo(*ghi*)perylene, was used.

Analysis of metals. Samples of 0.3 g pooled digestive glands and gills were used for metal analysis. Samples were digested in concentrated nitric acid (Baker's 65%). Digestion was performed in reaction flasks on a hot plate at 80°C for 24 h. After cooling, solutions were transferred and diluted with ultrapure water (NANOPURE II Barnstead) to a final volume of 10 ml. Determination of metal concentrations (Cu, Zn, Cd, Pb, Cr) was undertaken using an ICP-Mass (ELAN 5000) spectrophotometer (three replicates per analysis). In all experiments several blanks were processed to ensure that contamination was not occurring.

Biochemical analysis

Cytochrome P450 system. The pooled digestive glands of four to six mussels were used for each replicate sample, and five to seven replicate samples were prepared per site. Microsomal fractions were obtained at 4°C by differential centrifugation as described in Livingstone (1988). Frozen tissues were homogenized in 1:4 w/v 10 mM Tris-HCl, pH 7.6, 0.15 M KCl, 0.5 M sucrose. Microsomal fractions were diluted in 10 mM Tris-HCl, pH 7.6, 20% w/v glycerol at protein concentrations of $\sim$10 mg ml^{-1}, after consecutive centrifugations at $500g \times 15$ min, $10\,000g \times 45$ min and $100\,000g \times 90$ min. Biochemical measurements were carried out either immediately (cytochrome P450 content, NADPH cytochrome c (P450) reductase activity) or after overnight storage in liquid nitrogen (benzo[*a*]pyrene hydroxylase activity).

Total cytochrome P450 and '418-peak' (putative denatured cytochrome P450) contents were assayed by the carbon monoxide difference spectrum of sodium dithionite-reduced samples using an extinction coefficient of 91 mM^{-1} cm^{-1} for cytochrome P450. The 418-peak was quantified in arbitrary units as described in Livingstone (1988). NADPH-cytochrome c reductase activity was measured by the increase in absorbance of cytochrome c reductase reduction at 550 nm (extinction coefficient 19.6 mM^{-1} cm^{-1}) after adding NADPH to the microsomal fraction (Shimakata *et al.* 1972). Benzo[*a*]-pyrene hydroxylase (BPH) activity was assayed in the presence of NADPH by the fluorimetric assay of Dehnen *et al.* (1973) as described in Livingstone (1987). Assay conditions in a final volume of 1 ml were 50 mM triethanolamine-HCl, pH 7.6, 10 mM $MgCl_2$, 60 µM BaP (in 40 µl dimethylformamide), 0.2 mM NADPH and $\sim$1 mg microsomal protein. Reaction was started by the addition of benzo[*a*]pyrene and terminated after 10 min by 1 ml cold acetone. This assay measures predominantly phenols at the excitation/emission wavelengths of 467/525 nm.

Acetylcholinesterase (AChE) activity. AChE activity was assayed in the gills by a modification of the method of Ellman *et al.* (1961). The gills were homogenized in 1:5 (tissue weight:buffer volume) ice-cold 100 mM Tris-HCl, pH 8.0, centrifuged at $12\,000g \times 30$ min and the supernatant immediately used for AChE activity, in the presence of 1 mM acetylthiocholine and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB); the increase in absorbance was recorded at 405 nm. Total protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Metallothioneins (MT). MT concentrations were determined by spectrophotometric measurement of SH content as described in Viarengo *et al.* (1997). Frozen tissues (gills and digestive gland) were homogenized in 3 vols 0.5 m sucrose, 20 mM Tris-HCl, pH 8.6, containing 6 µM leupeptine and 0.5 mM PMSF (phenylmethyl sulphonylfluoride) as antiproteolytic agents, and 0.01% ß mercaptoethanol as a reducing agent. The homogenate was then centrifuged at $30\,000g$ for 20 min at 2°C to obtain a supernatant containing MTs. A two-step ethanol/chloroform precipitation of the supernatant was carried out. The resulting pellet was resuspended in 150 µl 0.25 M NaCl and 150 µl 1 N HCl containing 4 mM EDTA was added to the sample. This was followed by reaction with DTNB (5,5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M NaH_2PO_4/Na_2HPO_4, pH 8.0. Sample absorbance was read at 412 nm and MT concentration estimated using reduced glutathione as a reference standard.

Cellular analysis

Frozen digestive glands of 10 mussels per site were cut in a cryostat at a cabinet temperature of −24°C. Sections (8 m) were collected onto glass slides and stored at −40°C until use.

Lysosomes. Hystochemical measurement of the lysosomal marker enzyme, ß-glucuronidase, was carried out as described in Cajaraville *et al.* (1991). Sections were incubated at 37°C in a medium containing naphthol AS-BI-ß-d-glucuronide as substrate. Incubation lasted for 15 min since longer incubation times rendered excessive amounts of reaction product. After staining with diazonium salt solution, sections were fixed in Baker's formol calcium, and counterstained with a 0.1% aqueous solution of Fast Green FCF. Sections were then rinsed in distilled water and mounted in glycerol gelatin. Lysosomes appeared stained as purple-coloured discrete granules. ß-glucuronidase-stained sections were used to measure the structure of lysosomes in an automatic image analysis system (Cajaraville *et al.* 1991). Light microscopic images were acquired by a colour video camera and binary images segregating lysosomes from the rest of digestive cell cytoplasm were generated by assigning pseudocolours to critical gray levels. The image analysis programme measures the areas, perimeters, and numbers of lysosomes

with reference to digestive cell cytoplasm, and calculates the following parameters: lysosomal volume density ($VD = V_L/V_C$), lysosomal surface density ($SD = S_L/V_C$), lysosomal surface-to-volume ratio ($S/V = S_L/V_L$) and lysosomal numerical density ($ND = N_L/V_C$), where V is the volume, S is surface, N is number, L is lysosomes, C is digestive cell cytoplasm and D is density. Five measurements were made per section.

Peroxisomes. The histochemical detection of the peroxisomal marker enzyme catalase was performed as described by Orbea *et al*. (1999). Sections were fixed for 5 h in phosphate-buffered formaldehyde, pH 7.4, and incubated for 40 min in a medium containing 3,3′-diaminobenzidine and H_2O_2 as substrates in Teorell–Stenhagen buffer, pH 10.5. Sections were then rinsed, dehydrated in a graded series of ethanol and mounted in DPX. Peroxisomes appeared stained as brownish-coloured discrete granules. Catalase-stained sections were used to measure the structure of peroxisomes by applying a manual point counting method (Cajaraville *et al*. 1997). A lattice with 168 test points was superimposed onto the preparations with the aid of a *camera lucida* attached to a light microscope, and the fractions of test points falling onto peroxisomes and digestive epithelium were determined. Additionally, the diameter of 90 peroxisomes was measured in each section using a measuring paper superimposed on the preparation. Values of 0.5, 1 or 1.5 mm were assigned to each peroxisome, 1 mm being equivalent to 0.73 μm in the tissue section. The data obtained were used to calculate the same stereological parameters as for lysosomes.

Autometallography

Metals were determined in tissue sections by the method of Danscher (1984), as described by Soto and Marigómez (1997). The method is based on the autoinduced silver amplification of metal ions in biological sections. Cryostat sections were covered with an uniform thin layer of photographic emulsion under safety light conditions. After drying in completely dark conditions, sections were subsequently rinsed in developer (hydroquinone and sodium hydroxide 1:5), stop (1% acetic acid), and fixative bath (sodium thiosulphate 1:9). Sections were mounted in Kaiser's gelatine. Black silver deposits (BSD) indicate the presence of metal ions in the tissue section and were quantified as volume density (VD) of BSD in relation to digestive tubule volume by automated image analysis, as described above for lysosomal structure measurements. VD was defined as $VD = VB/VC$, where VB is the volume of black silver deposits and VC is the volume of digestive cell cytoplasm.

Statistical analysis

Results concerning metals, biochemical and cellular markers are presented as mean ± SEM. Significant differences between groups of values were established at the $p < 0.05$ level using Duncan's test. For cellular markers, data on VD and ND were logarithmically transformed before statistical analysis. Pearson's correlation coefficients were calculated and only $p < 0.05$ was accepted as significant.

Results

Chemical analysis

The PCB load of the different organisms was estimated on the basis of seven PCB congeners selected from the GC-ECD profile. These congeners were quantified separately, and results are given in table 1. Strong differences among sampling sites were observed. Mussels from Barcelona showed the highest PCB residues (260 ng g^{-1} w.w.), being 34-fold higher than those sampled in the Ebro Delta (8 ng g^{-1} w.w.). High levels of PCBs were also detected in mussels from Denia (132 ng g^{-1} w.w.). An analogous geographical profile of contamination was observed for DDTs, although differences among sampling sites were less striking.

The concentration of parent PAHs and their alkyl derivatives is reported in table 2. Differences among sampling sites were evidenced considering the sum of parental PAHs, but especially their alkyl derivatives. Mussels from Denia and Barcelona showed the highest PAH levels, with tissue residues 100-fold higher than those from the Ebro Delta and Cullera. Looking at PAH distributions, alkyl derivatives dominated over parental ones (78–90% of total PAHs) in Denia and Barcelona, whereas the opposite trend was observed in the other stations (26–37% of total PAHs). Trace amounts of the procarcinogen benzo[*a*]pyrene were detected in all the stations, but particularly in Barcelona (24 ng g^{-1} w.w.).

Table 1. Concentration of polychlorinated biphenyls (PCBs) and DDTs in the digestive gland of mussels sampled along the Spanish Mediterranean coast. Results are in ng g^{-1} wet weight. Each sample is a pool of four to five digestive glands.

	Barcelona	Ebro Delta	Alboraya	Cullera	Denia
PCB no.[a]					
28	8.0	–	–	–	–
52	10.5	2.1	3.0	1.5	4.0
101	46.4	0.9	3.6	1.9	12.3
118	30.0	0.4	2.0	1.2	6.1
153	52.1	2.2	6.6	5.1	62.1
138	98.0	2.0	8.2	5.2	40.2
180	14.7	–	0.3	0.2	7.7
ΣPCBs	259.7	7.6	23.7	15.1	132.4
pp′DDE	32.9	2.1	12.8	4.6	12.4
pp′DDT	5.9	0.1	0.5	0.4	2.9
ΣDDTs	38.8	2.2	13.3	5.0	15.3

–, Below detection limit.

[a] PCB-28: 2,4,4′-trichlorobiphenyl; PCB-52: 2,2′,5,5′-tetrachlorobiphenyl, PCB-101: 2,2′,4,5,5′- pentachlorobiphenyl; PCB-118: 2,3′,4,4′,5-pentachlorobiphenyl; PCB-138: 2,2′,3,4,4′,5′-hexachlorobiphenyl; PCB-153: 2,2′,4,4′5,5′-hexachlorobiphenyl PCB-180: 2,2′,3,4,4′,5,5′-heptachlorobiphenyl.

Table 2. Parental and alkylated polycyclic aromatic hydrocarbons (PAHs) in the digestive gland of mussels sampled along the western Mediterranean Coast. Results are given in ng g^{-1} wet weight. Each sample is a pool of four to five digestive glands.

	Barcelona	Ebro Delta	Alboraya	Cullera	Denia
Naphthalene (N)	0.2	–	–	–	0.2
C1-N	0.6	1.4	0.1	0.9	7.1
C2-N	0.9	1.7	0.1	1.4	28.0
Fluorene (F)	0.5	0.1	0.2	–	4.2
C1-F	28.5	0.2	0.4	0.2	104
C2-F	102	0.4	1.2	0.6	325
Phenanthrene (P)	9.1	0.3	0.6	0.1	52.2
Anthracene (A)	2.6	0.2	0.7	0.1	2.8
C1-P/A	96.4	0.5	3.0	0.6	404
C2-P/A	456	1.0	11.4	1.4	1092
Fluoranthene (F1)	34.3	0.8	5.0	1.1	30.0
Pyrene (P)	71.5	0.9	5.4	1.2	41.6
C1-F1/P	288	0.6	7.4	1.2	191
C2-F1/P	478	0.7	4.9	1.0	251
Benz(*a*)anthracene	29.3	1.2	6.7	0.9	11.0
Chrysene	106	1.4	8.7	1.4	65.7
Benzo(*b*)fluoranthene	50.1	2.0	10.5	1.6	29.2
Benzo(*k*)fluoranthene	28.3	1.9	9.6	1.7	8.6
Benzo(*a*)pyrene	23.6	2.2	7.0	1.0	4.9
Indeno(1,2,3-*cd*)pyrene	17.9	2.6	9.1	1.4	8.8
Dibenz(*ah*)anthracene	9.1	2.1	5.4	0.6	2.1
Benzo(*ghi*)perylene	21.9	2.3	9.0	1.1	11.8
Σ Parental PAHs	405	18.0	78.0	12.1	273
Σ Alkylated PAHs	1451	6.4	28.5	7.2	2402
Σ Total PAHs	1856	24.4	106.5	19.3	2675

–, Below detection limit.

Table 3. Mean concentrations of metals (Cu, Zn, Cd, Pb, Cr) in the digestive gland and gills of mussels (*Mytilus galloprovincialis*) sampled along the Spanish Mediterranean coast. Results are mean ± SEM ($n = 3$), in $\mu g\, g^{-1}$ wet weight. Samples from the Ebro Delta were not available for this study.

Metals	Barcelona	Alboraya	Cullera	Denia
Digestive gland				
Cu	8.50 ± 0.09	13.76 ± 0.90*	4.23 ± 0.36	9.36 ± 1.01
Zn	27.76 ± 2.14	19.91 ± 1.71	19.71 ± 1.61	26.95 ± 5.17
Cd	0.16 ± 0.04	0.18 ± 0.04	0.27 ± 0.04	0.19 ± 0.02
Pb	3.71 ± 0.25*	2.51 ± 0.27	0.91 ± 0.19	1.74 ± 0.14
Cr	2.82 ± 0.15	22.21 ± 1.29*	1.55 ± 0.28	1.74 ± 0.07
Gill				
Cu	3.39 ± 0.79#	2.28 ± 0.25#	1.80 ± 0.47	1.34 ± 0.04
Zn	4.25 ± 1.01	15.41 ± 3.25	18.10 ± 1.96	11.35 ± 1.75
Cd	0.02 ± 0.02	0.06 ± 0.01	0.11 ± 0.05	0.02 ± 0.01
Pb	2.03 ± 0.48	0.52 ± 0.27	1.20 ± 0.52	0.31 ± 0.02
Cr	0.33 ± 0.11	1.77 ± 0.03*	0.88 ± 0.01	0.29 ± 0.05

* Statistically significant differences respect to the other stations ($p < 0.05$).
\# Statistically significant differences respect to Denia ($p < 0.05$).

Concentrations of toxic and essential metals measured in digestive gland and gills are shown in table 3. Mussels from Alboraya showed extremely high levels of Cr in both gills and digestive gland, as well as elevated Cu concentrations. Elevated Pb concentrations were detected in Barcelona. In contrast, relatively constant Zn concentrations were observed in digestive glands (20–28 $\mu g\, g^{-1}$ w.w.) and gills (11–18 $\mu g\, g^{-1}$ w.w.) of mussels from all locations, except those from Barcelona which showed the lowest Zn concentration in gills. Cd levels were moderate ($< 0.3\, \mu g\, g^{-1}$ w.w.), with no significant differences among sampling sites.

Biochemical markers

Metallothioneins. MT content in digestive gland and gills is shown in table 4. In agreement with metal body burden, the highest MT levels were recorded in mussels from Alboraya, followed by those sampled in Barcelona harbour. MT content was higher in digestive gland than in gills, but still detectable in the latter.

Cytochrome P450 system. Cytochrome P450 system components and activities determined in the digestive gland of the studied mussels are given in table 5. Cytochrome P450-specific content was significantly elevated in mussels from the Ebro Delta (80 $pmol\, mg^{-1}$ protein) and no differences were observed among the other stations (57–67 $pmol\, mg^{-1}$ protein). An additional peak at ~418–420 nm appeared in all the spectra, and it was quantified in arbitrary units; the mean value

Table 4. Metallothionein levels in the digestive gland and gill of *Mytilus galloprovincialis*. Results are in $nmol\, g^{-1}$ wet weight. Values are mean ± SEM ($n = 4$). Samples from the Ebro Delta were not available for this study.

	Barcelona	Alboraya	Cullera	Denia
Digestive gland	19.89 ± 2.56	28.16 ± 1.47*	18.74 ± 1.68	14.83 ± 1.22
Gill	3.44 ± 0.46	4.92 ± 0.13*	2.01 ± 0.19	2.61 ± 0.18

* Statistically significant differences respect to the other locations ($p < 0.05$).

Table 5. Cytochrome P450 system determined in the digestive gland and acetylcholinesterase activity (AChE) in gills of mussels sampled at five stations along the western Mediterranean Coast.

	Barcelona	Ebro Delta	Alboraya	Cullera	Denia
Cytochrome P450 (pmol mg^{-1} protein)	56.8 ± 5.4	86.1 ± 6.1*	66.8 ± 9.9	60.1 ± 12.6	65.1 ± 10.6
'418-peak' (a.u mg^{-1} protein)	25.7 ± 1.8	25.8 ± 2.4	23.9 ± 2.1	23.3 ± 3.5	20.6 ± 0.6
NADPH cytochrome c reductase (nmol min^{-1} mg^{-1} protein)	24.6 ± 1.2	24.8 ± 1.6	20.7 ± 2.3	20.0 ± 2.5	21.7 ± 1.1
BPH (a.u. min^{-1} mg^{-1} protein)	151.3 ± 35.4	76.3 ± 35.6	133.6 ± 63.1	266.9 ± 56.9[e]	105.2 ± 23.0
BPH turnover (a.u. min^{-1} $pmol^{-1}$ P450)	5.9 ± 1.2	3.4 ± 1.8	5.6 ± 2.4	13.1 ± 3.7	5.22 ± 1.2
AChE (nmol min^{-1} mg^{-1} protein)	25.8 ± 3.9	25.7 ± 3.1	31.1 ± 1.6	23.6 ± 1.6#	29.9 ± 4.3

* $p < 0.05$ versus Barcelona; [e] $p < 0.05$ versus Ebro Delta; # $p < 0.05$ versus Alboraya.

of this peak was slightly higher in mussels from Barcelona and Ebro Delta than in the other locations. The same trend was observed for NADPH-cytochrome c(P450) reductase activity. BPH activity was significantly elevated in Cullera if compared with the reference site (Ebro Delta); high BPH activity was also recorded in Barcelona. When BPH was expressed as an estimate of turnover number (a.u. min^{-1} $pmol^{-1}$ P450), the highest values were again detected in mussels from Cullera, and the lowest in the Ebro Delta. The increased BPH activity and BPH turnover in Cullera corresponded with a relatively depleted AChE activity (table 5).

Cellular markers

Lysosomes. Changes in lysosomal structure were found among mussels collected at different sites (figure 2). Lysosomal volume density was significantly elevated in mussels from Barcelona and Denia (figure 2A), which also showed enhanced lysosomal surface density (figure 2B). Lysosomal surface-to-volume ratio, a parameter inversely related to size, was lower in mussels from Barcelona, Denia and Cullera with respect to those from the reference site (figure 2C). Finally, mussels from Barcelona showed higher lysosomal numerical density than those from other stations (figure 2D).

Peroxisomes. Results concerning digestive cell peroxisomes are given in figure 3. The most striking feature is that mussels from Barcelona showed higher number of peroxisomes than those from the other stations (figure 3D), and that their peroxisomes were smaller (higher surface-to-volume ratio; figure 3C). Nonetheless, no differences between stations were recorded in terms of volume or surface densities (figure 3A and B); peroxisomes of mussels from the Ebro Delta, Alboraya, Cullera and Denia had similar structure.

Autometallography. Results of the volume density of autometallographied black silver deposits (BSD) are given in figure 4. In agreement with metal data, the highest volume density of BSD was recorded in mussels from Alboraya. BSD correlated with reported MT levels ($r^2 = 0.78$), the former being an estimation of metal bioavailability in digestive gland lysosomes.

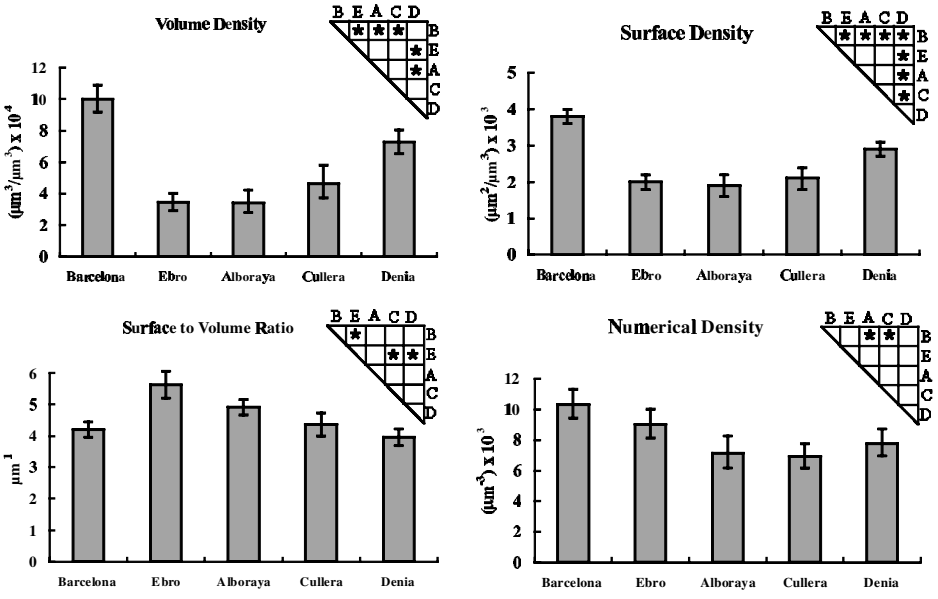

Figure 2. Structure of lysosomes in digestive cells of mussels sampled at five stations along the western Mediterranean coast. Lysosomal volume density, surface density, volume to surface ratio, and numerical density are indicated. Vertical segments show standard errors (95%, $n = 10$). Asterisks indicate significant differences ($p < 0.05$) between sampling sites according to the duncan's test. B, Barcelona; E, Ebro Delta; A Alboraya; C, Cullera; D, Denia.

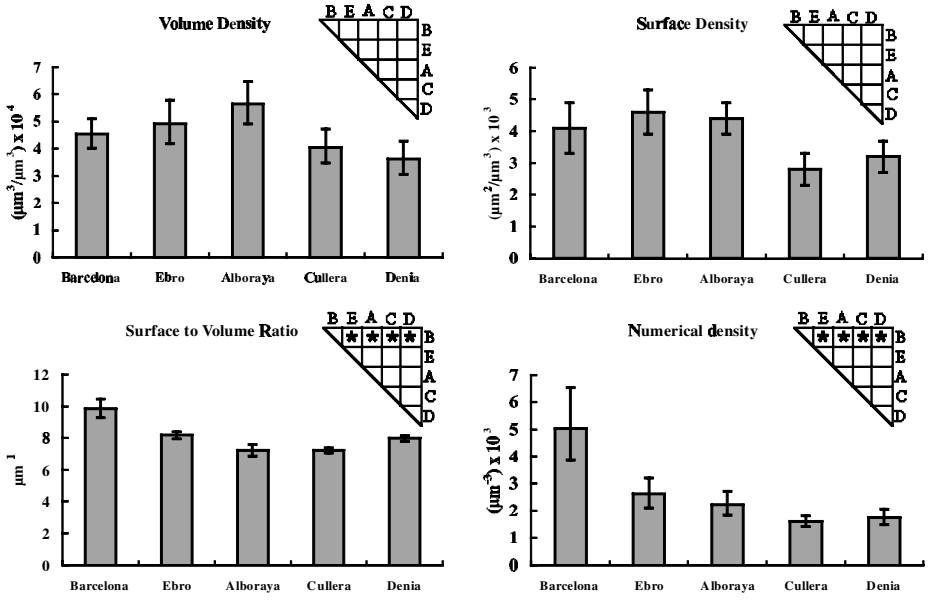

Figure 3. Structure of peroxisomes in digestive cells of mussels sampled at five stations along the western Mediterranean coast. Peroxisomal volume density, surface density, volume to surface ratio, and numerical density are indicated. Vertical segments show standard errors (95%, $n = 10$). Asterisks indicate significant differences ($p < 0.05$) between sampling sites according to the Duncan's test. B, Barcelona; E, Ebro Delta; A, Alboraya; C, Cullera; D, Denia.

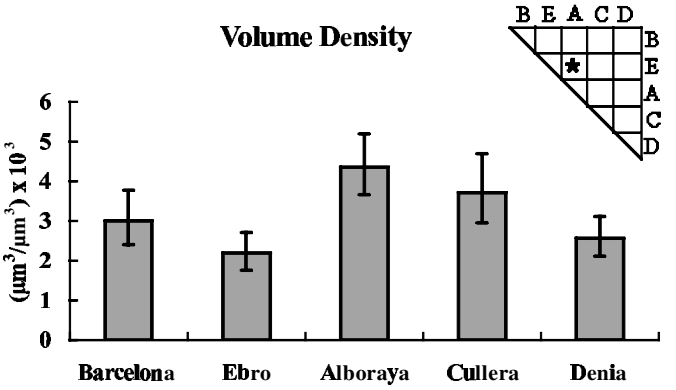

Figure 4. Volume density of autometallographied black silver deposits in digestive cells of mussels sampled at five stations along the western Mediterranean coast. Vertical segments show standard errors (95%, $n = 10$). Asterisks indicate significant differences ($p < 0.05$) between sampling sites according to the duncan's test. B, Barcelona; E, Ebro Delta; A, Alboraya; C, Cullera; D, Denia.

Discussion

The digestive gland in bivalve molluscs is the major site of accumulation of organic contaminants as well as toxic and essential metals (Widdows *et al.* 1983, Walsh and O'Halloran 1997). In the present study, there were marked differences among sites in terms of contaminants accumulated by mussels. Metals body burden was significantly higher in mussels from Alboraya (particularly Cr and Cu) and Barcelona (high levels of Pb). Consistently, the highest metallothionein content was observed in those organisms (table 4). Nonetheless, no statistically significant correlation between metals capable of MT induction (Cd, Zn, Cu, Pb) (Engel and Roesijadi 1987, Viarengo 1989), and MT content was observed. Only levels of Cr correlated with MT content ($r^2 = 0.87$); this metal has been reported to induce MT synthesis in mammal tissues (Fleet *et al.* 1990, Solis-Heredia *et al.* 2000), and the reduction of Cr (VI) to Cr (III) via reactive intermediates is thought to be part of its toxicity mechanism (Standeven and Wetterhan 1991).

Apart from metals, mussels were exposed to many other contaminants from the surrounding waters. In the present work, PAHs, PCBs and DDTs were monitored because of the high bioaccumulation by molluscs (PAHs), and its persistence and bioaccumulation along food chains (PCBs and DDTs) (Porte and Albaigés 1993). Although very high levels of PAHs and PCBs were detected in mussels from Barcelona and Denia, cytochrome P450 system components and BPH activity failed to indicate such an exposure. Inconsistent results have been reported over the last years regarding cytochrome P450 induction in mussels (Nasci *et al.* 1989, Michel *et al.* 1994, Solé *et al.* 1996, 1998, Porte *et al.* 2001). Elevation of mytilid BPH activity did not occur in every study, and when the activity increased, the magnitude of the response was of the order of 2–3-fold. Such levels of response (i.e. differences between putative clean and contaminated sites), the low activities commonly reported in mytilids, and the complexity of the system, which may be induced by certain pollutants and inhibited by others, may certainly account for the discrepancies on the use of BPH as a biomarker. Indeed, considering the complexity of the aquatic environment, and the coexistence of inducing and inhibiting chemicals, it is not unreasonable to indicate that Barcelona and Denia coastal areas are characterized by intense shipping traffic; thus, mussels are

exposed not only to high levels of PAHs, but also to tributyltin (TBT) from the antifouling paints (Morcillo *et al.* 1997), and TBT has been reported to inhibit cytochrome P450-catalyzed activities in molluscs (Morcillo and Porte 1997, Morcillo *et al.* 1998).

Despite the apparent lack of response to PAH exposure, the results of this study indicate elevated BPH activity in mussels from Cullera (3.5-fold higher activity than mussels from the reference site), which also exhibited the lowest AChE activity. AChE inhibiting pesticides (organophosphates and carbamates) have been shown to inhibit gills AChE in mussels (Escartín and Porte 1997); although other compounds (e.g. metals, PAHs) can inhibit this activity in fish (Payne *et al.* 1996, Kang and Fang 1997). One, among many, possible explanations for the coincidence of these two altered biomarkers may be related to the exposure of mussels to biocides used in orange tree areas located nearby.

In contrast to biochemical marker data, mussels from Barcelona and Denia did show significant changes of lysosomal structure (volume, surface, size) (figure 2). Long-term exposure to certain aromatic hydrocarbons and complex mixtures of petroleum hydrocarbons have been reported to cause lysosomal enlargement (Lowe *et al.* 1981, Cajaraville *et al.* 1995). In this work, a significant correlation between changes in lysosomal structure (particularly volume and surface density) and PAHs and PCBs residues was detected (figure 5). In addition, mussels from Barcelona showed higher number of lysosomes (figure 2D), probably as a consequence of high metal exposure, as indicated by Etxeberria *et al.* (1994). The good correlation between Zn and Pb residues in digestive gland and number of lysosomes ($r^2 = 0.64$ and 0.71 for Zn and Pb, respectively) agrees with this hypothesis.

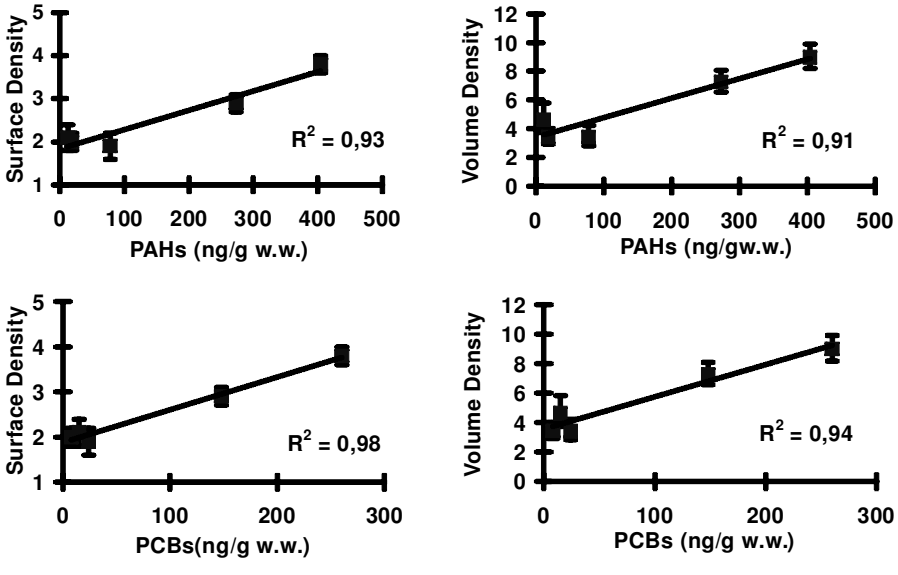

Figure 5. Correlation between lysosomal surface density (SD) and volume density (VD) and PAH and PCB residues detected in digetive gland of mussels.

The concentration of the procarcinogen PAH benzo(*a*)pyrene was particularly high in mussels from Barcelona ($24\,ng\,g^{-1}$). Despite of the absence of BPH induction, mussels from this station showed peroxisome proliferation (higher number and smaller peroxisomes than elsewhere). Benzo(*a*)pyrene exposure has been shown to cause induction of peroxisomal oxidases in mussels (Cancio *et al.* 1998). Other studies have reported an increase of the number of digestive tubules containing peroxisomes in mussels fed with a microencapsulated mixture of phenanthrene, fluoranthene and benzo(*a*)pyrene (Krishnakumar *et al.* 1997). In addition, peroxisomes of Barcelona mussels were smaller than elsewhere. Smaller peroxisomes together with peroxisome proliferation were detected in mussel from the Atlantic and related to PAHs/PCBs exposure (Orbea *et al.* 1999).

Table 6 summarizes the results obtained in the present study. Tissue residues of the studied chemicals do not always provide a valuable explanation for the increase/depletion of the biochemical and cellular markers. This further supports the hypothesis that not only the analysed pollutants, but also many others, and complex interactions among them and with the biological systems, would be responsible of the observed biological responses. Furthermore, factors other than pollutants (namely temperature, salinity, food availability, reproductive stage) might be sources of environmental stress, and can certainly have an influence on biochemical and cellular markers (Escartín and Porte 1997, Minier

Table 6. Summary of the results obtained for the battery of chemical, biochemical and cellular markers applied to the study. √indicates significant differences with respect to the reference site (E) for metals and biomarkers. For organic contaminants, statistics were not used, and √indicates higher levels (18–100-fold) than in the reference site.

	B	E	A	C	D
PAHs	√				√
PCBs	√				√
DDTs	√				
metals			√		
BaP hydroxylase				√	
AChE				√	
Metallothioneins			√		
Lysosomal VD	√				√
Lysosomal SD	√				√
Lysosomal S/V	√		√		√
Peroxisomes S/V	√				
Peroxisomes ND	√				
BSD					

B, Barcelona; E, Ebro Delta; A, Alboraya; C, Cullera; D, Denia.

et al. 2000). Nonetheless, despite the overall variation in mussel responses, some facts are discernible: (1) the higher levels of stress in Barcelona and Denia mussel populations, (2) the metal contamination problem in Alboraya, and (3) a non-identified stress source in the area of Cullera.

Finally, the evolution of the bivalve response with time is an essential aspect that has to be addressed in order to assess the robustness of the used markers. In this sense, it should be mentioned that a second survey carried out 3 months later focussing on biochemical markers contributed to further reinforce the above-mentioned observations. Hence, (1) Barcelona and Alboraya's mussels exhibited significantly higher MT levels, namely 20.4–23.9 nmol g^{-1} wet weight against 12 nmol g^{-1} w.w. in Cullera and Denia (Martinez *et al.* 1998), and (2) a strong BPH induction was again detected in Cullera's mussels (3-fold), followed by Barcelona (1.9-fold) and Denia (1.7-fold) in comparison with the reference site (Ebro Delta) (Solé and Porte, unpublished data).

In summary, this study provides further support for the use of a battery of biomarkers as a tool to assess the health of coastal areas. The set of biomarkers used indicates cellular stress in mussels from certain areas; it is unclear though if the stress is elicited as an adaptational response to a polluted environment or if it is a sign of impaired health, and this is certainly one of the main limitations of the use of biomarkers in pollution biomonitoring. The approach can be improved in the future by integrating population dynamics into field studies, what will led to a stronger diagnostic evidence of pollution impact.

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