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# Assessment of biological effects of environmental pollution in Mersin Bay (Turkey, northeastern Mediterranean Sea) using *Mullus barbatus* and *Liza ramada as* target organisms

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ABSTRACT

The increasing emphasis on the assessment and monitoring of marine ecosystems has revealed the need to use appropriate biological indicators for these areas. Enzyme activities and histopathology are increasingly being used as indicators of environmental stress since they provide a definite biological endpoint of pollutant exposure. As part of an ecotoxicological assessment of Mersin Bay, EROD enzyme activity and histopathological response in selected organs and tissues of two species of fish, *Mullus barbatus* (red mullet) *and Liza ramada* (thinlip grey mullet), captured from area were examined. Pollutant (Organochlorines (OC), alkylphenols (APs) and BPA) levels and biomarker responses in tissue samples were evaluated together for their potential to alter the metabolism and cellular aspects in liver and gonad. Elevated induction of EROD activity and histopathological alterations in contaminated samples from Mersin Bay was observed compared to reference site indicating the exposure to potential pollutants.



## 1. Introduction

Coastal area of Mersin Bay, situated in northeastern Mediterranean Sea, is the recipient of organic pollutants from a variety of sources such as sewage outfalls, industrial wastes, maritime shipping, oil transportation and agricultural activity from adjacent urban areas. Pollution originating from Mersin Bay may not cause common acute effects in aquatic organisms, however, long term exposure to these pollutants can lead to chronic effects and accumulation in food chain by the time.

The OC compounds persist in the environment for a long period of times. They can accumulate in fat tissue and pass through the food chain and they tend to cause wide range of toxicity. Due to their tendency of bioaccumulate and biomagnify, the effects of these chemicals on animals are a matter of concern. Fish living in coastal areas have often been used as target organism for monitoring the pollution as they concentrate in their tissues. Several studies and biomonitoring programs conducted in the area have revealed the presence of OCs (PCBs, DDT and its metabolites, OC pesticides) in Mersin Bay. These pollutants referenced in the literature have been reported in fish tissues (*Mullus barbatus*, *Mugil cephalus*) at various concentrations (MEDPOL reports of Tugrul et al., 2005, 2007, 2008, 2009; Yemenicioglu, 2003; Yemenicioglu et al., 2004, 2006).

In their studies, Gedik and Imamoğlu (2011) reported that low levels of PCBs and Aroclors in sediment indicated that there is no ecotoxicological risk for marine environment in Mersin Bay. But the integration of pollutant analysis together with biochemical and cellular responses should be considered for evaluating both the fate of pollutants and their impact on the biota. However, endocrine disrupting effects of these OC compounds on fish is not reported in Mersin Bay. Beside OCs, information on the levels of APs and BPA in biota are not also available.

An effective monitoring system using biochemical markers has been established to demonstrate the xenobiotics in the environment. The cytochrome P450 system has proved to be a very suitable tool for biochemical and environmental monitoring. It is particularly sensitive to a broad spectrum of industrial contaminants (e.g

*Abbreviations:* OP, 4-t-octylphenol; NP, 4-n-nonylphenol; PCB, polychlorinated biphenyls; BPA, bisphenol-A; PAH, polycyclic aromatic hidrocarbon; EROD, ethoxyresurufin-o-deethylase; OC, organochlorine; AP, alkylphenol; BSTFA, Bis(-trimethylsilyl)trifluoroacetamide; TMCS, trimethylsilyl chloride; SMI, Scaled Mass Index; FCF, Fulton's Condition Factor.

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dioxins, PCB, PAH) (Havelkova et al., 2007). Ethoxyresorufin-O-deethylase (EROD) is functionally linked to cytochrome P450 system. This enzyme transforms substrates into products emitting a measurable fluorescent signal and is more sensitive than the determination of CYP 450. Regarding the biochemical responses of organisms to most aquatic pollutants, EROD has a major role in oxidative metabolism and is recognized as a biomarker of exposure of contaminants. Among toxicity mechanisms, oxidative stress, defined as an injuries effects due to cytotoxic reactive oxygen species, causes oxidative damage to tissues. This occurs mainly in the endoplasmic reticulum of liver cells, where cytochrome P450 (CYP) activities may generate reactive oxygen species (ROS) as byproducts of detoxification processes (van der Oost et al., 2003)). ROS can inflict irreversible damage to cell constituents, either lethal or carcinogenic. In OC-contaminated organisms, CYP1A expression is increased, which tends to elevate ROS levels in cells (Schlezinger et al., 2006). This ROS production adds on to that of the mitochondrial electron transport chain (Cadenas and Davies, 2000). Liver cells showed increasing EROD levels, probably reflecting the induction of CYP1A by some of the PCB congeners. An increased ROS production by CYP1A activity would require higher activities of antioxidant enzymes such as catalase, superoxide dismutases and glutathione peroxidases (Lemaire et al., 2010).

Numerous field studies demonstrated significant and strong increase of hepatic EROD activity in *M. barbatus* (Porte et al., 2002) and *Liza ramada* (Mihailovic et al., 2006) from PCB-polluted marine environment. They have found good relation between PCB and EROD activity in fish tissue.

Histopathological alterations in liver and gonad tissues induced by OCs and APs have been used as supporting parameters in several studies. Ovaries of red mullet from Ionian areas (Gallipoli and Porto Cesareo) exhibited an abnormal space between cytoplasm and oocyte envelope (Corsi et al., 2002). Melanomacrophage centers in testes and disturbances in gonad and connective tissue in red mullet (*M. barbatus)* of Cortiou area from French coast were reported (Zorita et al., 2008; Martin-Skilton et al., 2006). In carp (*Cyprinus carpio*), BPA caused severe alterations in gonad structure such as lost of typical lobular appearance in testis and oocyte atresia (Mandich et al., 2007). Fibrosis in testis, increase in picnotic nucleus and ovarium atresia in 4-NP exposed *Danio rerio* were reported (Weber et al., 2003).

The purpose of this study was an assessment of biomarker responses in two fish species. Considering OCs, APs and BPA have a potential to induce alterations in fish tissues, liver and gonad ultrastructure of contaminated *M. barbatus* and *L. ramada* was also observed and compared with fish collected from reference site.

## 2. Materials and methods

### 2.1. Sample collection and preparation

Red mullet (*M. barbatus*) and thin lip mullet (*L. ramada*) were collected between January 2010 and February 2012 at 10–20 m depth by local fisherman using trawl and seine. Specimens from the same species were also collected from the reference area, 100 km away from the center of Mersin, where sediment OCs, APs and BPA were typically low (Fig. 1).

Mature individuals were chosen for analysis and their genders were determined by macroscopic observations of gonads. Once caught fish were anesthetized with phenoxyethanol (0.4 ml/L) and their length and weight were measured. These morphometric measurements were used to calculate individual body condition using the scaled mass index (M) reported by Peig and Green (2009). This index is based on the equation $M = M_i(L_a/L_i)^{bsma}$, where $M_i$ and $L_i$ are the individual weight and length, respectively, $L_a$ is the average length of all individuals, and $b_{sma}$ is the quotient between the slope of the regression of the Ln-transformed body weight on the Ln-transformed length of each individual. Fulton's condition factor (K) was calculated according to the equation (body weight)/(body length)$^3 \times 100$, where W is the weight expressed in g and L the length in cm (Nash et al., 2006). Morphometric measurements and condition indices were given in Table 1. Livers and gonads were quickly dissected and hepatosomatic index [HSI = (liver weight/body weight) $\times$ 100] was calculated. Subsamples of tissues were taken for electron microscopic examination and analysis of EROD and pollutants.

### 2.2. Extraction and clean-up

Extraction and derivatization were performed according to the procedure found elsewhere with small modifications (Khim et al., 1999; Li et al., 2001). Briefly, liver samples of individuals per location were pooled and freeze-dried. Samples of approximately 5 g were then homogenized by grinding in a mortar and soxhlet-extracted with 300 ml hexane:dichloromethane (1:1) for 18 h. Solvent extract was evaporated to 1 ml under gentle stream of $N_2$ and cleaned up by adding 1–2 ml concentrated $H_2SO_4$. Resultant hexane layer was collected and dried with $Na_2SO_4$ for removal of excessive water. Hexane extract was then evaporated to 1 ml for fractionation.

Extracts of 1 ml were passed through 15 g activated florisil (60–100 mesh, Sigma–Aldrich, 46,385) column for cleanup and fractionation. First fraction, eluted with 75 ml of 100% hexane, contained PCBs and DDE. DDT and DDD in second fraction were eluted with 100 ml of hexane:dichloromethane (4:1). Remaining APs, NP, OP and BPA were eluted in third fraction using 100 ml of dichloromethane:methanol(4:1). Fractions, contain OCs and APs and BPA, were evaporated to 1 ml. 100 μl aliquot of BSTFA (with 1% TMCS) was added to third fraction followed by vigorous shaking for 60 s at room temperature for derivatization. 100 μl of water was then added to hydrolyze excess unreacted BSTFA. 1 g $Na_2SO_4$ was added to remove water. Solution was transferred to another vial and remaining residue was collected by successive rinsing with dichloromethane. Solution was again evaporated to 1 ml under gentle stream of nitrogen.

### 2.3. Instrumental analysis

Individual PCB congeners (18, 28, 31, 44, 52, 101, 118, 138, 149, 153, 170, 180, 194, 209) and DDT and its metabolites, DDD and DDE were quantified using gas chromatography-electron capture detector. A fused capillary column with HP5 phase (Agilent J&W, 19091J-413), 30 m $\times$ 0.32 mm in diameter, with a thickness of 0.25 μm, was used. The oven temperature was held at 70 °C for 2 min, then elevated to 260 °C at 3 °C/min and held at 260 °C for 30 min. The recoveries of surrogate standard 2,4,5,6 tetrachloro-m-xylene were 92 ± 12%. Quantitation was performed using external standard calibration mixture (Accustandard, AE-00061). For each Aroclor (1254 and 1260), nine peaks were selected to quantify the amount of that Aroclor.

Liver alkylphenols (NP and OP) and BPA were determined using a Agilent 7890 GC interfaced to Agilent 5975C mass spectrometer with HP5-MS column (Agilent J&W). The column oven temperature was programmed from 60 to 290 °C at a rate of 3 °C/min, with a final holding time of 24 min. The ion source and the analyzer were maintained at 250 °C. The selected ions used for monitoring were 179 and 292 for NP, 135 and 206 for OP and 357 and 358 for bisphenol-A. NP, OP and bisphenol-A were identified and quantified by comparison of retention times and spectra of standard compounds (Accustandard, M-1626). Recoveries were obtained

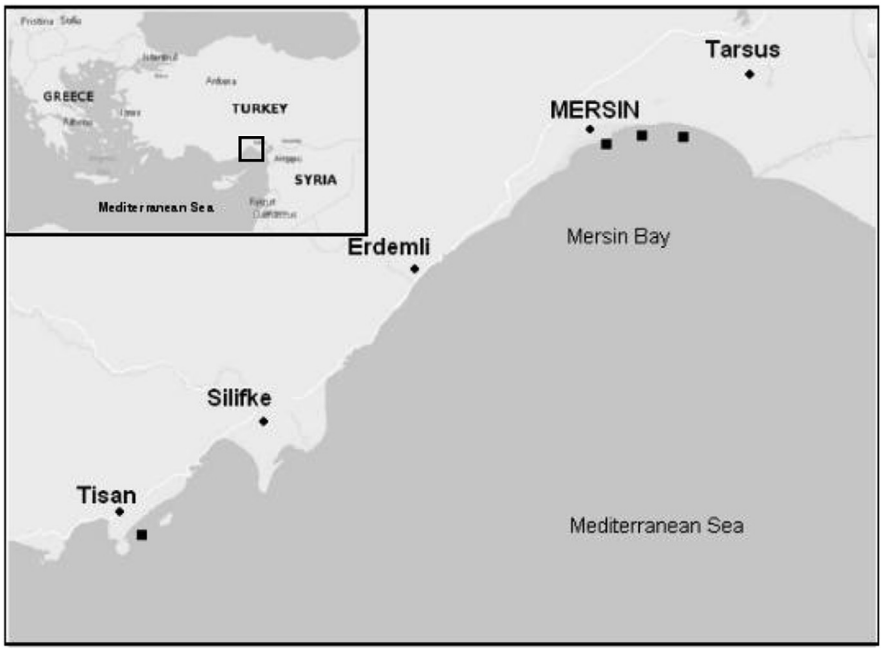


**Fig. 1.** Map of the northeastern Mediterranean Sea showing the sampling sites of *Mullus barabatus* and *Liza ramada*, representing by squares. Tisan was used as reference location (∑DDT and ∑PCB were below 0.1 ng/g d.w. APs and BPA; n.d., Kalay et al., 2012).

**Table 1**
Mean and associated standard deviation of the biological variables measured in fish species collected from Mersin Bay and reference site. SMI; scaled mass index, FCF; Fulton's condition factor.

| Species | Area | Length | Weight | SMI | FCF |
|---|---|---|---|---|---|
| *Mullus barbatus* | Mersin Bay | 16.97 ± 1.44 | 58.58 ± 15.56 | 54.38 ± 2.81 | 1.16 ± 0.09 |
| | Reference site | 16.30 ± 1.27 | 53.96 ± 13.58 | 56.09 ± 3.28 | 1.22 ± 0.06 |
| *Liza ramada* | Mersin Bay | 26.67 ± 3.58 | 145.22 ± 65.87 | 139.95 ± 0.09 | 0.72 ± 0.05 |
| | Reference site | 27.05 ± 3.71 | 154.39 ± 70.84 | 146.32 ± 13.07 | 0.73 ± 0.07 |

with using internal standards Acenaphthene $d_{10}$ and Phenanthrene $d_{10}$ (Accustandard, M-1626-IS) as 104 ± 9%, 87 ± 11% and 91 ± 13% for NP, OP and bisphenol-A, respectively.

The quantification limits were defined as three times the procedural blank values and the limit of detection was calculated as three times the noise level of chromatogram. The limit of quantification of the present study was 15, 3, 50, 0.02 and 0.1 ng/g for NP, OP, BPA, DDT and metabolites and PCBs, respectively.

### 2.4. EROD analysis

Preparation of postmitochondrial supernatant (PMS) from tissue samples was performed according to Nilsen et al. (1998) with some modifications. Briefly, partially defrosted liver samples (about 0.1 g) were homogenized at 4000 rpm for 5–6 turn with a homogenizer with teflon pestle in an ice cold phosphate buffer (pH 7.4). During homogenization, tubes were cooled with a small container filled with ice. Homogenate was centrifuged at 12,000 g for 20 min at 4 °C. The resultant supernatant was immediately frozen and stored in liquid nitrogen until analysis.

Excitation and emission wavelength of spectrofluorometer (Varian Cary Eclipse) were set to 535 and 585 nm, respectively. Enzyme assay was performed in a temperature controlled cuvette with a peltier unit. Resorufin production was measured with mixing of 1960 μl of EROD buffer (phosphate buffer, pH 7.4), 10 μl 7-ethoxyresorufin (Sigma, E3763) (0.4 mM) and 20 μl PMS. The reaction was initiated by the adding of 10 μl NADPH (Sigma, N1630) (100 mM). Resorufin formation during reaction was measured over 2–3 min by recording the change in fluorescence. Finally, 10 μl resorufin (Sigma, R3257) was added to solution as an internal standard and increase in fluorescence was recorded.

Activity was calculated by using the following formula;

$$\text{pmol resorufin/min/mg protein} = F_s/t \times R/F_R \times 1/V_s \times 1/C_s$$

where $F_s/t$ is increase in fluorescence per minute, R is amount of resorufin added, increase in fluorescence of resorufin, $V_s$ is sample volume and $C_s$ is protein concentration in sample (mg/ml). Protein concentration was measured according to Bradford (1976).

### 2.5. Electron microscopy

For the ultrastructural study, 1 $mm^3$ slices of liver and gonad samples were primarily fixed in 2.5% gluteraldehyde solution for 4–6 h and postfixed in $OsO_4$. Following fixation, tissue was dehydrated in a graded ethanol-propylene oxide series and embedded in resin. Thin sections (70 nm) were contrasted with uranyl acetate and lead citrate and were then transferred to copper grid (300 mesh) for studying in a transmission electron microscope (Jeol Jem 1011).

### 2.6. Data analysis

Significant differences in EROD enzyme activities between

**Table 2**
Concentrations of DDT compounds, PCBs, alkylpenols and BPA in fish liver samples (ng/g dw). Mean values ± standard deviation. N.D.; not detected (<limit of detection). (PCB 28 coeluted with 31 and PCB 138 coeluted with 149).

| | *Liza ramada* n = 10 | *Mullus barbatus* n = 10 | *Liza ramada* reference n = 3 | *Mullus barbatus* reference n = 3 |
|---|---|---|---|---|
| Lipid (%) | 24.1 ± 4.3 | 15.5 ± 3.2 | 19.6 ± 3.0 | 15.2 ± 1.6 |
| DDE | 579.61 ± 481.76 | 316.31 ± 64.11 | 15.74 ± 7.38 | 0.29 ± 0.22 |
| DDD | 21.32 ± 9.12 | 3.77 ± 2.02 | 1.21 ± 0.17 | <0.02 |
| DDT | 1.11 ± 0.53 | 0.78 ± 0.38 | 0.41 ± 0.15 | <0.02 |
| **ΣDDT** | 602.04 ± 491.41 | 320.85 ± 66.51 | 17.37 ± 0.34 | 0.29 ± 0.22 |
| 18 | 4.35 ± 1.52 | 3.03 ± 0.63 | N.D. | N.D. |
| 28 + 31 | 15.23 ± 2.21 | 12.08 ± 4.93 | <0.1 | <0.1 |
| 52 | 17.91 ± 13.12 | <0.1 | <0.1 | <0.1 |
| 44 | <0.1 | <0.1 | N.D. | N.D. |
| 101 | 18.74 ± 12.23 | 21.33 ± 7.71 | <0.1 | <0.1 |
| 118 + 149 | 11.48 ± 9.89 | 7.61 ± 3.76 | <0.1 | <0.1 |
| 153 | 1.53 ± 1.12 | <0.1 | <0.1 | <0.1 |
| 138 | 19.00 ± 28.32 | <0.1 | <0.1 | <0.1 |
| 180 | 2.77 ± 1.66 | <0.1 | N.D. | N.D. |
| 170 | 7.50 ± 4.55 | <0.1 | <0.1 | <0.1 |
| 194 | 8.78 ± 4.68 | <0.1 | <0.1 | <0.1 |
| 209 | <0.1 | <0.1 | N.D. | N.D. |
| **ΣPCB** | 107.28 ± 79.30 | 44.91 ± 17.80 | <0.1 | <0.1 |
| OP | <3 | <3 | N.D | N.D. |
| NP | 52.73 ± 28.38 | 21.64 ± 10.53 | N.D. | N.D. |
| BPA | <50 | <50 | N.D. | N.D |

**Table 3**
EROD activity in *Liza ramada* and *Mullus barbatus* collected from Mersin Bay and reference site (Tisan).

| | | EROD pmol/min/mg protein |
|---|---|---|
| (*Liza ramada*) N = 22 | min | 22.21 |
| | max | 93.13 |
| | Mean ± std dev | 64.21 ± 23.24 |
| *Liza ramada* reference N = 14 | min | 19.09 |
| | max | 31.21 |
| | Mean ± std dev | 26.25 ± 3.73 |
| *Mullus barbatus* N = 22 | min | 23.36 |
| | max | 468.18 |
| | Mean ± std dev | 182.85 ± 108.17 |
| *Mullus barbatus* reference N = 16 | min | 12.76 |
| | max | 93.55 |
| | Mean ± std dev | 52.59 ± 23.96 |

Mersin Bay and reference site were determined using nonparametric Mann–Whitney U test. To test the relationship between biomarker responses and pollutant levels, multiple pairwise correlations (Spearman correlation) were applied to data. Analysis of covariance (ANCOVA) was used to compare enzyme activity and pollution parameters (∑PCB and ∑DDT) between sites. This method compared regression lines by testing the effect of a categorical factor on a dependent variable (y-var) while controlling for the effect of a continuous co-variable (x-var, covariate). The categorical factor (2 sites) splits the relationship between x-var (log weight as covariate) and y-var (EROD, ∑PCB or ∑DDT as dependent variable) into several linear equations, one for each level of the categorical factor. Firstly, interactions among the covariate and categorical factors were tested: if these are significant, they indicate that the slopes are not homogeneous and so the parallelism assumption of the standard ANCOVA is not satisfied. All statistical analyses were performed with R statistics (GNU project).

**Table 4**
Spearman correlation coefficients (r) and their p values for the relationships between condition indices, EROD enzyme activity and pollutant levels. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

| | EROD | SMI | FCF | ΣDDT | ΣPCB |
|---|---|---|---|---|---|
| ***Mullus barbatus*** | | | | | |
| EROD | 1 | | | | |
| SMI | −0.49* | 1 | | | |
| FCF | −0.16 | 0.20 | 1 | | |
| TDDT | 0.70** | −0.41 | −0.22 | 1 | |
| TPCB | 0.83*** | −0.63** | −0.14 | 0.65** | 1 |
| ***Liza ramada*** | | | | | |
| EROD | 1 | | | | |
| SMI | −0.54* | 1 | | | |
| FCF | −0.53* | 0.46 | 1 | | |
| TDDT | 0.57* | −0.72** | −0.36 | 1 | |
| TPCB | 0.80*** | −0.68** | −0.51 | 0.70** | 1 |

## 3. Results

### 3.1. Bioaccumulation of pollutants

The major pollutants identified in fish liver tissue contain DDT and its metabolites, PCBs, alkylphenols and BPA. The levels of components are shown in Table 2. The GC-ECD profiles of the extracts showed the occurrence of PCB congeners in liver tissue of both fish species. Higher concentrations were observed in *L. ramada*. Their results for ∑PCB and ∑DDT were approximately 2-fold higher than *M. barbatus*. Differences in concentrations of PCBs and DDT compounds were also apparent between fish samples collected from Mersin Bay and reference site. In the reference site, these organic contaminants were detected in very low concentrations, were below the detection limits. Similar pattern were

**Table 5**
Intercept, slopes and determination coefficients ($R^2$) of the linear regression models linking EROD, ΣDDT and ΣPCB to fish weight obtained after log-transformed of the raw data. N.S; not significant.

| *Mullus barbatus* | | | | | | *Liza ramada* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Intercept | Slope | $R^2$ | p | Intercept | Slope | $R^2$ | p |
| Mersin Bay | EROD | 0.236 | 1.113 | 0.64 | <0.001 | 1.456 | 0.175 | 0.16 | N.S. |
| | TDDT | 1.224 | 0.675 | 0.65 | <0.05 | −3.482 | 2.496 | 0.53 | <0.05 |
| | TPCB | −0.133 | 0.993 | 0.59 | <0.05 | −0.195 | 0.733 | 0.22 | N.S |
| Reference site | EROD | −0.645 | 1.318 | 0.34 | <0.05 | 0.511 | 0.421 | 0.64 | <0.05 |
| | TDDT | −1.274 | 0.850 | 0.26 | N.S. | −0.890 | 0.9667 | 0.71 | <0.01 |
| | TPCB | −0.064 | 0.071 | 0.04 | N.S. | −0.275 | 0.161 | 0.37 | N.S. |

observed in profiles of DDT and its metabolites. The values of ∑DDT calculated for both fish samples collected from Mersin Bay, were as high as 320.85–602.04 ng/g dry weight, 35–1000 times higher than values calculated for samples collected from reference site.

OP and BPA were below the quantification level in fish samples collected from the both Mersin Bay and reference site. NP were only detected in samples and higher concentrations were measured in liver samples of *L. ramada* collected from Mersin Bay.

### 3.2. EROD enzyme activity

A nonparametric Mann–Whitney test was used to test the null hypothesis that there was no significant difference between EROD activity in fish samples collected from Mersin Bay and reference site. Therefore, enzyme activity was significantly higher in fish samples collected from contaminated area than those of reference site ($p < 0.05$, Table 3). This difference was also observed between fish samples collected from contaminated and reference site. Enzyme activity was detected at higher levels in samples of *L. ramada* and *M. barbatus* (64.21 and 182.85 pmol/min/mg protein, respectively) collected from contaminated area than those of reference site.

### 3.3. Data analysis

EROD activity, condition indices (SMI and FCF) and pollutant concentrations (ΣDDT and ΣPCB) were correlated after pooling data from two sites (Table 4). EROD was positively correlated with ΣDDT and ΣPCB and was significant for both species. Correlation between SMI and pollutants levels was significant for *L. ramada*. But the relationship was significant between ΣPCB and SMI for *M. barbatus*. There was no significant correlation between FCF and other parameters. To identify natural variables that possibly influence EROD activity in both fish species, we tried to regress EROD on fish body weight (Table 5). EROD activity showed a significant association with body weight of *M. barbatus* but was not significantly associated with weight of *L. ramada*. Significant relationship was found for the regression of pollutant levels (ΣDDT and ΣPCB) on weight for *M. barbatus*. But the association of these pollutants with fish weight was not significant for *L. ramada*. The interaction of fish weight (covariate in ANCOVA) with site was not significant for EROD and pollutant parameters indicating that the slopes of the relationships between biomarker/pollutants and fish weight were the similar among sites.

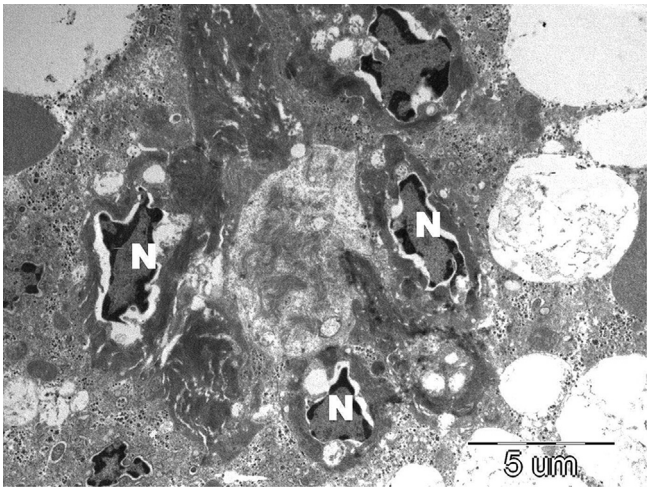


**Fig. 2.** Hepatocyte ultrastructure of Mullus barbatus showing apoptotic cell and nucleus (N). (X6000).

### 3.4. Electron microscopy

#### 3.4.1. Mullus barbatus

A histopathological evaluation was done for liver and gonad tissues of both fish species to determine the existence of tissue toxicity during their exposure to contaminants. Hepatic parenchyma of *M. barbatus* showed appearance of apoptotic cell and nuclei (Fig. 2). The gonad of each animal was examined for histopathologic alterations. An expansion of perinuclear region in apoptotic cells, congestion with numerous erythrocyte in veins, breakdown of organization and synchronization of spermatogenesis and delayed development of reproductive cells were observed in male samples of *M. barbatus* (Fig. 3). Histological sections of female gonads of *M. barbatus* displayed numerous myelin figures in perivitelline space and multivesicular bodies and large vacuoles in follicular cell cytoplasm (Fig. 4).

#### 3.4.2. Liza ramada

Swelling of hepatocyte mitochondria, dissolution of matrix and cristae were observed in *L. ramada* (Fig. 5). Testes of *L. ramada* exhibited chromatin defects in testicular spermatids and myelin figures in Sertoli cells (Fig. 6). Similar large vacuolation was observed in oocytes of *L. ramada*. Apart from this common alteration, degenerated cell debris, disorganization and loops in follicular basal lamina, swelling of mitochondria, dissolution of matrix and cristae in cytoplasm of follicular cell were also observed in *L. ramada* (Fig. 7). As a general statement, ovarium alterations were more pronounced in *L. ramada* than in *M. barbatus*.

## 4. Discussion

### 4.1. Bioaccumulation of pollutants

Considering the levels of contaminants in animal tissue, 14 analyzed PCB congeners and DDT compounds were detected in most samples of liver of *L. ramada and M. barbatus*. However, differences were found among samples. Table 2 shows that samples

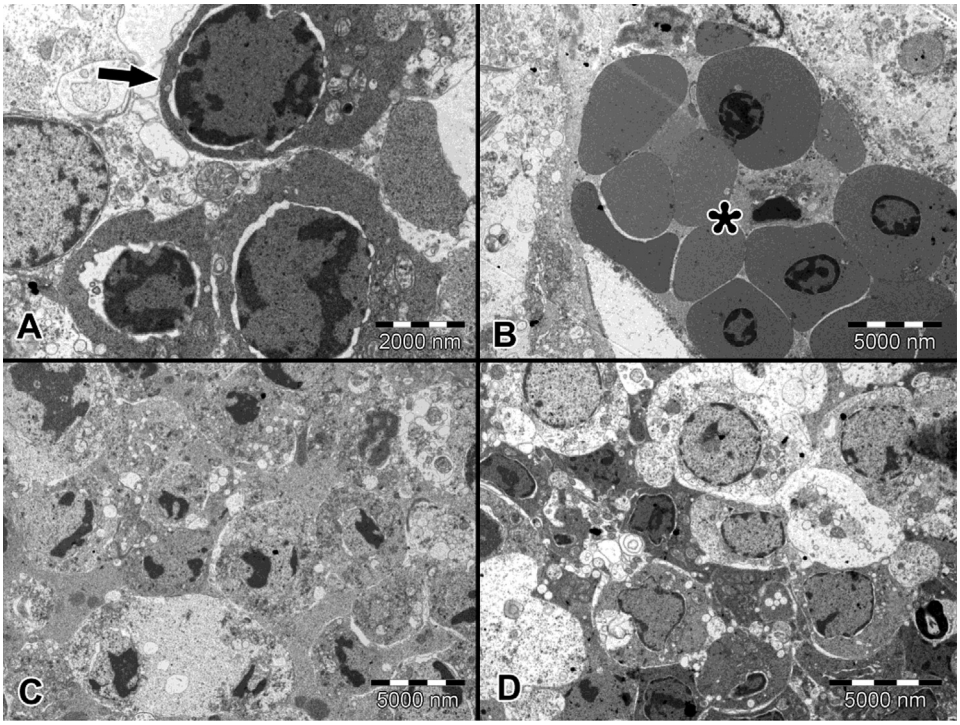


**Fig. 3.** Testes of Mullus barbatus. A. Enlargement of perinuclear space in apoptotic cells (arrow), B. Congested veins (star), C and D. Disorganized spermatogenesis and delayed development of germ cells. (A: X12000, B and C: X5000, D: X6000).

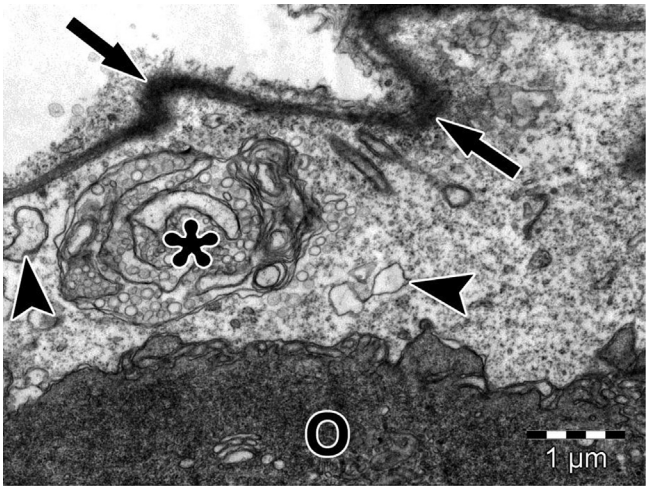


**Fig. 4.** Oocyte of Mullus barbatus showing vacuoles (arrow head), multivesicular bodies (star) and loopy basal lamina (arrow) in cytoplasm (O). (X20000).

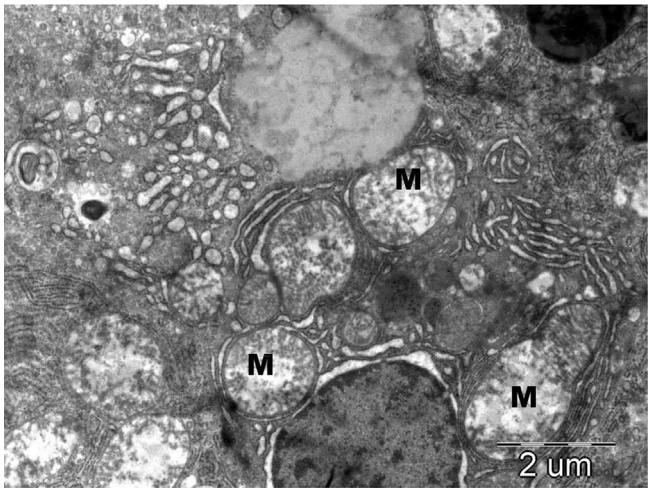


**Fig. 5.** Hepatocyte ultrastructure of *Liza ramada*. Cytoplasm shows swelling in mitochondria (M) and dissolution of matrix and cristae. (X 12,000).

from Mersin Bay exhibited higher levels of contaminants than those from reference site. PCB (28 + 31), 52, 101, (118 + 149) and 138 were dominant congeners in all samples collected from Mersin Bay. Differences were also apparent among fish species. The highest levels of PCBs were found in *L. ramada* and can be attributed to the higher lipid content of this species when compared to *M. barbatus* (Table 2). Positive correlations between organochlorines and lipid content have already been reported (Coelhan et al., 2006; Felipe-Sotella et al., 2008; Trocino et al., 2009). As a catadromous species, *L. ramada* has the capacity to osmoregulate in freshwater (Cardona et al., 2008). It has been reported that the hyperosmosicity of the lacustrine fish facilitate the uptake of lipophilic compounds, so that pollutant levels in freshwater or estuarine fish are usually higher than marine fish (Oliver and Niimi, 1988). Due to their benthic feeding strategy, mullets (Mugilidae) tend to accumulate more pollutants than other fish species (Ortiz-Zarragoitia et al., 2014). Furthermore, this species is found in more contaminated areas (Kottelat and Freyhof, 2007) and is more tolerant to coastal pollution (Boglione et al., 2006).

As for PCBs, the levels of total DDTs detected in the liver indicated apparent differences among fish species, showing 2-fold increase in *L. ramada*. The tissue concentration levels in Table 2 can be considered representative of pollution in Mersin Bay. PCB levels reported in sediments of Mersin Bay (Gedik and Imamoğlu, 2011) supports the relationship of the *L. ramada* and *M. barbatus* pollutant

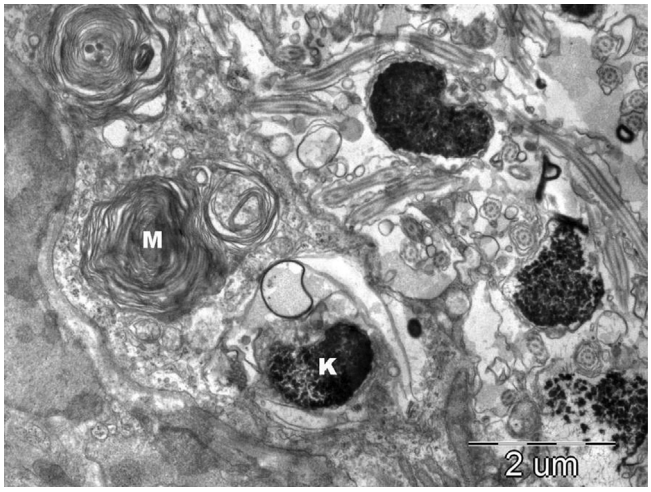


**Fig. 6.** Histopathology of testes of *Liza ramada* displaying miyelin figures (M) and degenerated chromatins (K). (X15000).

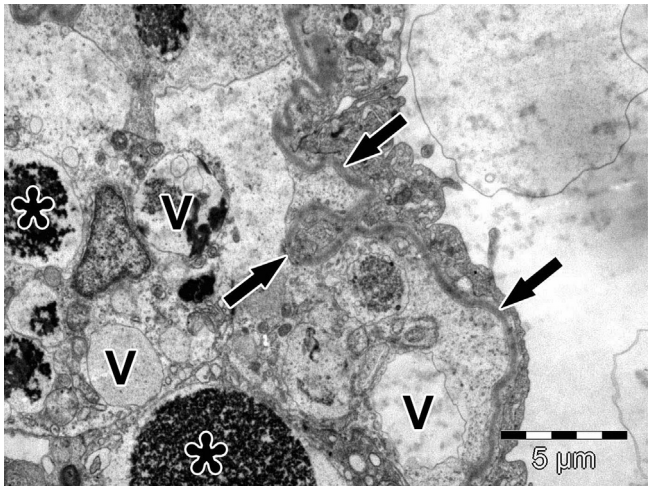


**Fig. 7.** Oocyte of *Liza ramada* showing vacuoles (V), loops in follicular basal lamina (arrow) and degenerated cell debris (star). (X7500).

uptake with the benthic concentration of PCBs. The total DDT content of the *M. barbatus* from Mersin Bay was almost similar to that of reported earlier values (Baştürk et al., 1980), indicating no apparent decline of this contaminant. In case where DDTs are still being detected in recent sediment and biota samples from Mersin Bay, it is associated with their environmental persistence and extensive use in the past and/or recent possible use of dicofol mixture in the area.

NP was the only alkylphenol compound that was measured above limit of detection and it was detected at levels higher than 10–20 times than OP. This indicates the wider use of NP in the area. NP levels with an average of 21 ng/g d.w. for *M. barbatus* and 52 ng/g d.w. for *L. ramada* are comparable with the reported values. Fish samples from Adriatic Sea (Ferrara et al., 2008) and Singapore (Basheer et al., 2004) were found to have NP concentrations ranged from 5 to 60 ng/g w.w and OP concentrations ranged from 0.2 to 31 ng/g d.w.

BPA was detected in fish samples including samples with below limit of quantification. No previous data was available for BPA contamination for marine biota of Mersin Bay. However, levels were lower than the fish samples from Singapore, at 66 ng/g w.w (average) (Basheer et al., 2004) and 250 ng/g d.w. (average) in flounder (*Platichthys flesus*) from Wadden Sea (Staniszewska et al., 2014) but comparable to the levels (0.8–6 ng/g w.w.) in fish from the coast of Italy (Mita et al., 2011) and (not detected – 69 ng/g d.w.) in fish species from coastal waters of Malaysia (Santhi et al., 2012).

### 4.2. EROD activity

Liver EROD activity revealed to be the responsive biomarker in this study due to significant differences between contaminated and reference area in both fish species (Mann–Whitney U test $p < 0.05$, Table 3). EROD activity in *L. ramada* and *M. barbatus* was 2.5 and 3-fold higher, respectively than in fish species collected from corresponding reference site. EROD activity in both fish species caught from reference site was in accordance with the reported values from other reference areas (Corsi et al., 2002; Della Torre et al., 2010 and Martinez-Gomez et al., 2012).

In our study, the highest enzymatic activity was recorded in *M. barbatus* sample (468 pmol/min/mg protein), is lower than that of reported for *Liza saliens* (1293 pmol/min/mg protein) and for *Solea vulgaris* (2000 pmol/min/mg protein) collected from heavily contaminated Izmir Bay, Turkey (Arınç and Şen, 1999). EROD activity values between 3 and 57 times above the minimum value have been recorded in fish species exposed to contaminants in laboratory and in field studies. Elevated EROD activities were obtained in fish species treated with PCBs in laboratory. Hugla and Thome (1999) reported a 4-fold induction of EROD activity in *Barbus barbus* and Nault et al. (2012) reported an 18-fold increase in *Oncorhincus mykiss*. Combined mixtures of PCB and NP treated Ictalurus punctatus showed 12-fold induced enzyme activity compared with the control group (Rice et al., 1998). However, controversial results have been reported from laboratory studies. Olsvik et al. (2009) found that the Atlantic cod *Gadus morhua*, exposed to 50 µg $l^{-1}$ BPA, showed 2.6-fold decrease in EROD activity. Such elevations in activity have also been fourf-1981 nd in different geographical areas and species. Au and Wu (2001) reported a 3-fold induction in samples from highly contaminated area of East Sha Chau (Hong Kong). 25 and 57-fold increase in activity of *M. barbatus* has been observed in samples caught from contaminated sites of western Mediterranean (Porte et al., 2002 and Corsi et al., 2002; respectively). Masmoudi et al. (2011) recorded high levels of EROD activities (28-fold higher than control) in *Liza aurata* collected from Tunis Bay. It has been suggested that EROD values over tissue upper limit of the baseline is indicative for exposure of planar organic compounds such as dioxins, furans, coplanar PCBs and some PAH compounds (Lyons et al., 2010). In our study, Tisan area was characterized by the low levels of contaminant concentrations in fish liver tissue. Therefore, it can be used as a reference area for biomonitoring studies. Mean EROD activity was almost 2.5 and 3-fold higher than reference site, indicating an elevation in EROD enzyme activity. Thus, induced EROD activity, the biochemical indice, in the fish samples collected from Mersin Bay suggest that this area contaminated by PCBs, DDTs, alkylphenols and possibly other contaminants such as dioxins, furans and PAHs.

### 4.3. Relationship between biomarker and pollutants

Also, correlations between EROD, condition indices and pollutant levels indicate that the pollutants (ΣDDT and ΣPCB) have an important role in altering the EROD activity levels (Table 4). The levels of pollutants and EROD enzyme activity increased significantly with fish weight (as shown by the slope and p-values in the ANCOVA table, Table 5). The response of biomarker and pollutant levels showed almost homogeneous slopes but different intercept values were observed. This indicates that biomarker responses and

pollutant levels in Mersin Bay and reference site affected at the same rate but in different magnitude.

### 4.4. Electron microscopy

#### 4.4.1. Mullus barbatus

*4.4.1.1. Liver.* The liver tissue showed many histopathological alterations. This could be expected as the liver have the ability to metabolize and detoxify exogenous compounds like xenobiotics, metals and toxins. It is therefore a target organ for various toxic substances. The histopathalogical analysis of *M. barbatus* hepatic tissue reveals the existence of some alterations such as apoptotic cell and nuclei which is characterized by the presence of condensed chromatin at the nuclear periphery, vacuolization in cytoplasm and degradation of cytoplasmic organelles (Fig. 2). The ultrastructural modifications observed in contaminated *M. barbatus* samples are similar to but less significant than those detected in other species exposed to various contaminants.

Hepatocellular vacuolation is frequently observed in fish exposed to pollution, for example, in english sole (*Pleuronectes vetulus*) (Au, 2004) and flathead grey mullet (*M. cephalus*) (El-Bakary and El-Gammal, 2010; Pinto et al., 2010; Padmini and Rani, 2011). This histological change may be an indication of some biochemical lessions, such as inhibition of protein synthesis and energy depletion (Pinto et al., 2010). The presence of apoptotic liver cell and nuclei mentioned here is also observed, after exposure of pollutants in field and laboratory, as for example in Atlantic bluefin tuna (*Thunnus thynnus*) (Corriero et al., 2013), brown bullhead catfish (*Ameriorus nebulosus*, 100 μM OP exposure) (Toomey et al., 1999) and medaka (*Oryzias latipes* 100 ppb NP exposure) (Weber et al., 2002). Vascular congestion, observed in liver tissue of *M. barbatus*, has often been reported in field and laboratory studies (Marchand et al., 2009; Padmini and Rani, 2011; Barja-Fernandez et al., 2013). Nuclear changes such as chromatin condensation and marginalization detected in liver hepatocytes is evident that the nucleus is an acting site of toxicants. Such nuclear alterations in fish liver cell have been reported in laboratory-based contaminant exposure of isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*) (Strmac and Braunbeck, 2000) and *Sparus aurata* (Traversi et al., 2014).

*4.4.1.2. Gonads.* The ultrastructual histopathology of testes was another indicator to reflect the damage of environmental pollutants. Similar to liver, apoptotic cell structures were observed in testes of *M. barbatus* (Fig. 3). Apoptosis was characterized by the expansion in perinuclear region. Delayed gonadal maturation and the lack of synchronization in maturation were also detected in male *M. barbatus* samples collected from contaminated sites of Mersin Bay. Delayed maturation were observed in *M. barbatus* samples collected from French-Italian coast in which the high levels of alkylphenols measured in bile (Martin-Skilton et al., 2006) and PCB and other organochlorine compounds measured in muscle in earlier studies (Porte et al., 2002). We did not find any case of intersex character in specimens. This could be attributed to low levels of pollutants in Mersin Bay in comparison with the other polluted areas. Unlike testes, ultrastructure of oocytes exhibited no severe abnormalities. Myelin-like structures in perivitelline space and multivesicular bodies and vacuoles in cell cytoplasm were seen in specimens of *M. barbatus* (Fig. 4).

#### 4.4.2. Liza ramada

*4.4.2.1. Liver.* In relation to *L. ramada*, histopathological alterations were more apparent in hepatocytes compared with *M. barbatus*. Swelling in mitochondria, dissolution of matrix and cristae and numerous lipofucsin granules in hepatocyte cytoplasm were observed in specimens sampled from Mersin Bay (Fig. 5). There are a variety of conditions in which hepatic lipofucsin granules has been associated with exposure to pollutants, and examples include *Solea ovata* exposed to benzo(a)pyrene (Au et al., 1999), neotropical fish exposed to biodegradable detergents (Pereira et al., 2014), *L. ramada* exposed to atrazin (Biagianti-Risbourg and Bastidae, 1995), *Catostomus commersonii* treated with bleached-craft pulp mill effluent (Couillard and Hodson, 1996), *Cynoglossus macrolepidotus* with high levels of burden of PAHs and PCBs collected from contaminated sediments of Hong Kong (Au and Wu, 2001) and *Zosteriosessor ophiocephalus* collected from contaminated Venice Lagoon (Nesto et al., 2007). Lipofucsins are end-product of the lipid peroxidation process (Viarengo et al., 2007). Therefore, this indicates the occurrence of oxidative stress in liver cell. Good correlation between elevated liver EROD activity and lipofucsin granule accumulation was observed in liver tissues of *S. ovata* upon laboratory exposure to benzo(a)pyrene (Au et al., 1999). Although histopathological alterations are indicative of general stress response and EROD induction is spesific response to toxic chemical, biochemical responses to contaminants can be indicator of histopathological changes. Endothelial cells are rich in CYP1A and it is well established that CYP1A induction, leading the EROD induction, can result in rupture in these cells and subsequent tissue damage (Ortiz-Delgado and Sarasquete, 2004).

*4.4.2.2. Gonads.* Ultrastructural lessions observed in male gonads of *L. ramada* involved primarily Sertoli cells (Fig. 6). Thus, myelin figures and remnant bodies within the cytoplasm of the Sertoli cells could be originated from phagocytozed and degenerated spermatids and spermatozoa. Abnormal packaging of chromatin was the characteristic of degenerated spermatids. It has been reported that severe effects of nonylphenol exposure on testicular structure and Sertoli cells have been observed for *Clarias gariepinus* (Sayed et al., 2012), *Zoerces viviparus* (Christiansen et al., 1998) and *Xiphophorus maculatus* (Kinnberg et al., 2000), resulted in impairment of spermatogenesis. Singh et al. (2008) have also reported reduced sperm motility in catfish *Heteropneustes fossilis* under the conditions of γ-HCH, DDT and chlorpyrofos exposure. Damaged Sertoli cells were recorded as a result of organochlorine pesticide endosulfan exposure in *Chiclosoma dumerus* (Da Cuna et al., 2011).

In our study, histopathological alterations were observed in gonads of female *L. ramada* from Mersin Bay: vacuolization in oocyte cytoplasm, enlargement of perivitelline space and presence of cell debris within this space, loops in follicular basal lamina, mitochondrial swelling and dissolution of matrix and cristae (Fig. 7). Faustino et al. (2010) suggested that the perivitelline space protects the embryo against environmental conditions and enlargement of this area is associated with increased oocyte degredation (Mikkelsen and Lindenberg, 2001). In embryos of mice, enlargement of perivitelline space have been reported in earlier studies (Nair et al., 2014).

## 5. Conclusion

Our results on the tissue contents of the contaminant body burden as well as biomarker responses in *L. ramada* and *M.* barbatus indicate that the study area is contaminated by anthropogenic chemicals. On the other hand, low levels of contaminants and EROD activity in the liver of fish species collected from the area of Tisan provided that this area can be used as a reference site for future studies. PCBs, DDTs, APs and BPA are not the only xenobiotics in the environment and thus cannot be solely responsible for the health effects on fish populations. Further investigations, both field and laboratory should therefore be conducted to determine the levels of other contaminants such as PAHs, dioxins and furans and their

possible histopathological effects on tissue. Finally, the biochemical results were confirmed by histopathological examinations where environmental pollutants induced hepatic and gonadal damage manifested by ultrastructural alterations.

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