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Transcriptional and post-transcriptional response of drug-metabolizing enzymes to PAHs contamination in red mullet (*, Linnaeus, 1758*): a field study

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1 **Transcriptional and post-transcriptional response of drug-metabolizing enzymes to PAHs**
2 **contamination in red mullet (*Mullus barbatus*, Linnaeus, 1758): a field study**

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27 **Abstract**

28 Aim of this study was to evaluate the responsiveness of red mullet (*Mullus barbatus*) liver
29 detoxification enzymes to PAHs at transcriptional and post-transcriptional levels in the field. Fish
30 were captured in the north-eastern Adriatic Sea, close to an oil refinery. Sixteen PAHs (EPA) were
31 determined in sediments and fish fillets; transcription levels of *cyp1a*, *cyp3a* and *abcc2* genes and
32 EROD, BROD, B(a)PMO, BFCOD, GST and UDPGT enzymatic activities were measured. Levels
33 of PAHs in sediments reflect the oil pollution gradient of the area, with weak correspondence in fish
34 fillets. *cyp1a* gene transcription and EROD, B(a)PMO and BFCOD activities were significantly
35 induced in the oil refinery site, and a slight up-regulation of *cyp3a* and *abcc2* was also observed.
36 GST and UDPGT remained unchanged. The present study provides the first data on detoxification
37 responses at transcriptional levels in the liver of red mullet and confirms phase I enzymes as
38 suitable biomarkers of exposure to PAHs in field studies.

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42 **Keywords:** *Mullus barbatus*, PAHs, CYP450, MRP2, GST, UDPGT

43 **1 Introduction**

44 Petroleum and its derivatives are the major and most widespread type of contaminants in marine
45 coastal areas. Several man-dependant sources are heavy contributors: shipping activities, industrial
46 and urban run-off, oil production and oil refining. Petroleum-related hydrocarbons in the marine
47 environment cause great concern due to their persistence in sediment and their toxicity for marine
48 organisms. Polycyclic aromatic hydrocarbons (PAHs) and their metabolites have the highest
49 toxicity, being mutagenic, cytotoxic and potentially carcinogenic (IARC, 1989). PAHs in the water
50 column and sediments cause a series of harmful effects in vertebrates and invertebrates, including
51 genetic damage, immune and endocrine dysfunction, malformations, fibrosis and cancer (Aas et al.,
52 2000). These effects determine a decrease in the survival capacity of resident species and thus a
53 reduction in biodiversity (Shailaja and D'Silva, 2003; Martin-Skilton et al., 2006a; Venturini et al.,
54 2008). Information about PAH bioavailability and the biological responses of organisms, such as
55 mechanisms of cell response, are therefore essential to assess how dangerous these contaminants are
56 in the environment and devise adequate strategies of environmental protection.

57 The primary biological system for detoxifying/bioactivating PAHs is the cytochrome P450
58 (CYP450) system (Aas et al., 2000; Jewett et al., 2002; Lee and Anderson, 2005), which consists of
59 several multigenic families of structurally and functionally related heme-proteins (Goksøyr and
60 Förlin, 1992). In fish, two gene subfamilies, namely *cyp1a* and *cyp3a*, encode for key CYP450
61 enzymes that are known to play a major role in the biotransformation of several compounds
62 including marine pollutants. Response of *cyp1a* towards PAHs has been extensively studied and is
63 known to be regulated by the aryl hydrocarbon receptor (*AhR*) (Stegeman and Hahn, 1994; Hahn,
64 1998). On the other hand, while *cyp3a* is known to be involved in the metabolism of several drugs
65 (Hasselberg et al., 2008; Christen et al., 2009), its role and regulation in fish is still matter of debate.
66 Few studies have directly addressed its involvement in the response to environmental contaminants,
67 and field data are lacking altogether. Moreover, species-specific differences in substrate specificity
68 towards different compounds, including β -naphthoflavone, were recently reported (Smith and
69 Wilson 2010). Enzymatic activities generally associated with CYP3A include benzyloxy-4-
70 [trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) (Hasselberg et al. 2008; Christen et al.
71 2009), testosterone hydroxylase (James et al., 2005), aminopyrine *N*-demethylase and erythromycin
72 *N*-demethylase (Vaccaro et al., 2007), with some contradictory results with regards to their
73 modulation in response to xenobiotics (Li et al., 2008).

74 UDP-glucuronyl transferase (UDPGT) and glutathione-S-transferase (GST) are major enzymes
75 involved in phase II of detoxification. Their response to environmental pollutants, including PAHs,

76 seems less pronounced than those of phase I, making them less suitable biomarkers of exposure in
77 fish (van der Oost et al., 2003; Martinez-Gomez et al., 2009).

78 Among ATP-binding cassette transport proteins, the multi-resistance protein 2 (MRP2), encoded by
79 the *abcc2* gene, excretes conjugated metabolites of phase I and II from cells, and is therefore known
80 as phase III of detoxification (Leslie et al., 2005). The *abcc2* sequence has already been identified
81 in some fish species (Sauerborn et al., 2004; Miller et al., 2007; Zaja et al., 2008; Zucchi, 2009) but
82 only few studies investigated its modulation by environmental pollutants. Significant induction of
83 *abcc2* gene transcription has been reported in Emerald rock cod (*Trematomus bernacchi*) exposed
84 to B(a)P (Zucchi, 2009). Similarly Paetzold et al. (2009) reported a significant increase in *abcc2*
85 transcription in killifish (*Fundulus heteroclitus*) from a PAH-contaminated area (Sydney Tar Ponds,
86 Nova Scotia, Canada).

87 Despite past and recent data on the modulation of detoxification proteins in response to PAHs,
88 significant interest is now summoned by the possibility to link phases I, II and III starting from gene
89 transcription profile (Bard, 2000; Paetzold et al., 2009). Characterization and expression profiling
90 of specific genes, such as *cyp1a*, *cyp3a* and *abcc2*, known to be involved in responses to marine
91 pollutants in selected fish species, can therefore be extremely important in environmental studies to
92 clarify cell detoxification pathways occurring in the natural environment.

93 Red mullet (*Mullus barbatus*) have been recommended as a species suitable for use as a
94 bioindicator in Mediterranean marine coastal areas (UNEP RAMOGE, 1999). The species is a
95 territorial benthic fish with well-known ecological and physiological characteristics and pronounced
96 sensitivity to different kinds of pollutants. Red mullet tends to accumulate pollutants to a greater
97 extent than other species (Zorita et al., 2008) and has therefore been used as a bioindicator for
98 pollution monitoring of polychlorinated biphenyls (PCBs), PAHs, alkylphenols and metals (Insausti
99 et al., 2009; Harmelin-Vivien et al., 2009; Ausili et al., 2008; Martin-Skilton et al., 2006b). While
100 pollutant-induced enzymes such as 7-ethoxyresorufin-*O*-deethylase (EROD), benzo(a)pyrene
101 monooxygenase (B(a)PMO), GST and UDPGT have been widely studied in this species (Regoli et
102 al., 2002; Corsi et al., 2002; Porte et al., 2002; Burgeot et al., 1994), no data is currently available,
103 to our knowledge, on the modulation of toxicologically relevant genes at the transcription level.

104 Aim of the present study was to evaluate the responsiveness of red mullet liver detoxification
105 enzymes to PAHs in the field at transcriptional and post-transcriptional levels. The link between
106 phases I, II and III of detoxification in fish in response to exposure to PAHs was also discussed.
107 Specimens of red mullet were captured in three sites with different degrees of PAH contamination:
108 sediment samples, as well fish fillets, were analyzed for PAHs content. Transcription of *cyp1a*,

109 *cyp3a* and *abcc2* genes and activities of EROD, benzyloxyresorufin-*O*-deethylase (BROD),
110 B(a)PMO, BFCOD, GST and UDPGT were assessed in fish liver.

111

112 **2 Methods**

113

114 2.1 Fish and sediment sampling

115 Specimens of red mullet were captured in November 2007 in the north-eastern Adriatic off a stretch
116 of coast characterized by high human impact, extending from 43.62816°N, 13.44080°E to
117 43.68213°N, 13.31534°E. Three sites with different degrees of PAHs contamination were
118 considered: one site was directly off an oil refinery (high-impact site HIS) (43.64152°N,
119 13.40688°E). The refinery occupies an area of 70 ha, processes 3.9 million tons of crude oil per
120 year and produces 2 million MWh/y. Storage capacity (about 1,500,000 m³) is among the largest in
121 Europe. All the crude oil arrives by sea and refinery products leave by sea (30%) and land (70%).
122 The many transport and processing operations lead to frequent accidental spills (the most recent of
123 fuel oil in April 2007). The other two sites were at increasing distances from HIS, one defined as
124 moderate impact (MIS) affected by urban run-off (43.66269°N, 13.34753°E) and the other as
125 reference (REF) (43.67660°N, 13.33383°E). The area normally has slow sea currents (2.5 m/sec)
126 flowing NW-SE direction, i.e. from the REF to the MIS and HIS sites. The sites were in a 7-Km
127 stretch of sea about 1300 m from the shore. Trawling was carried out for 900 m at depths between
128 20-30 m.

129 Twenty specimens were collected at each site: HIS specimens were 11.5-13.5 cm TL and weighed
130 20-45 g, MIS specimens were 11-13 cm TL and weighed 20-40 g, REF specimens were 12-14 cm
131 TL and weighed 26-40 g. All specimens showed recrudescence gonads at the time of sampling,
132 impairing sex determination. Nevertheless previous studies failed to observe any sexual
133 dimorphism for phases I and II in November (Mathieu et al., 1991).

134 Liver and muscle were immediately excised on board. Liver was flash frozen in liquid nitrogen and
135 stored at -80°C until molecular and biochemical analysis; muscle was stored at -20°C until chemical
136 analysis. At the same time, 25 cores of superficial sediment (0-10 cm) were obtained in the three
137 sites at a depth of 20 m and stored at -20°C until chemical analysis.

138

139 2.2 PAH analysis

140 The sixteen most toxic PAHs listed by the EPA were determined in all sediment samples and in
141 four pools of five fish fillets for each site. Five grams of sediment and 5 g of fish fillet were
142 extracted (Dionex mod. ASE 200 accelerated solvent extractor, Sunnyvale, USA) according to US-
143 EPA (1996) method 3545A and quantified by high-performance liquid chromatography (HPLC)

144 (Waters mod. 474 SFD and 996 PDA detectors, Milford, Massachusetts). Quantitative analysis was
145 done against a three-point linear calibration of PAH solution, obtained by dilution of the certified
146 standard mixture *TLC 16-PAH mix* (Supelco). Satisfactory linearity was obtained, with values of the
147 correlation coefficient R above 0.99. Detection limits, estimated as 3σ (IUPAC criterion) for each
148 PAH compound ranged from 0.01 to 0.5 ng g⁻¹. Certified reference materials, procedural blanks and
149 replicate samples were used as quality control procedures, and their reproducibility and recovery
150 were high (>85%). Precision, evaluated in terms of repeatability of the experimental results ($N=10$)
151 for the analysis of a real sample and expressed as relative standard deviation, ranged from 4.3%
152 (DBA) to 18.5% (NAPH) and was below 10% in most cases.

153

154 2.3 Molecular analysis

155 RNA was isolated from 50 mg of liver samples using the Fatty and Fibrous tissue kit (Biorad, USA)
156 according to the manufacturer's instructions and including a DNase treatment. RNA concentrations
157 were measured using a Shimadzu spectrophotometer at 260 nM. RNA quality was confirmed on 1%
158 agarose gel that showed discrete 18S and 28S rRNA bands.

159 cDNA for RT-PCR was generated with 0.5 µg total RNA from all samples in 20 µl reaction volume
160 using iScript cDNA Synthesis Kit according to the manufacturer's protocol (Biorad, USA).

161 Partial sequences for *cyp1a* and *cyp3a* genes were obtained by RT-PCR. *cyp1a* sequences of
162 leaping mullet (*Liza saliens*) (AF072899.1) and gilthead sea bream (*Sparus aurata*) (AF005719.1)
163 were aligned and primers were designed from highly conserved regions. *cyp3a* primers were
164 designed using sequences from European sea bass (*Dicentrarchus labrax*) (DQ268535.1) and
165 rainbow trout (*Oncorhynchus mykiss*) (AF267126). RT-PCR was performed in an Applied
166 Biosystem Thermal Cycler; the PCR mix contained 1 µl cDNA and 1 µl of Forward and Reverse
167 primers 10 µM, in 50 µl total volume. The cycling parameters were: 2 min denaturation at 94°C, 35
168 cycles at 94°C for 30 s, annealing at 55°C for 45 s, elongation at 72°C for 1 min, and 7 min final
169 extension at 72°C. Reaction products were separated by electrophoresis on 1.5% agarose gel in
170 TBE buffer, stained with ethidium bromide. PCR products were carefully excised from agarose gel
171 and purified with a QIAQUICK Gel Extraction Kit (Qiagen, Germany). PCR products were
172 sequenced by MWG (Germany) and confirmed to be the desired gene fragment by NCBI BlastN.

173 Real-time PCR was used to evaluate *cyp1a*, *cyp3a* and *abcc2* gene expression profiles. Primers with
174 an annealing temperature of 55°C were designed using IDTDNA www.idtdna.com. *16S* rRNA was
175 used as housekeeping gene. Each amplification reaction contained 12.5 µl SYBR Green mix, 1 µl
176 cDNA and 1 µl of Forward and Reverse primers 10 µM in 25 µl total volume. The cycling
177 parameters were: 10 min denaturation at 95°C, 40 cycles at 95°C for 30 s, annealing at 55°C for

178 45 s, elongation at 72°C for 1 min. All primer pairs gave a single peak of dissociation in all
179 reactions, and no amplification occurred in reactions without template. PCR efficiencies for each
180 primer pair were determined from a standard curve using dilutions of pooled cDNA ($R^2 > 0.97$ for
181 all primers; efficiencies *cyp1a* 99.6%, *cyp3a* 98.2 %, *abcc2* 105%, *16S* 109%). Primer sequences
182 used for RT-PCR and qPCR are reported in Table 1. Data were analysed by the $\Delta\Delta C_t$ method as
183 described by Pfaffl (2001) using Rest 2008 V2.0.7 software, that includes a correction for reaction
184 efficiency. The mathematical model used is based on the correction for exact PCR efficiencies and
185 the mean crossing point deviation between two group(s) (HIS and MIS) and the reference group
186 (REF). Subsequently the expression ratio results of the investigated transcripts are tested for
187 significances by a randomisation test (<http://www.gene-quantification.de/rest.html>).

188

189 2.4 Biochemical assays

190 Liver cytosolic and microsomal fractions were obtained as described by Corsi et al. (2003).
191 Microsomal EROD and BROD activities were measured according to the fluorimetric methods of
192 Burke and Mayer (1974). Assay conditions in the reaction mixture (final volume 2.25 ml) were as
193 follows: 50 mM Tris-HCl, 25 mM MgCl₂, 125 μ M NADPH and 50 μ l of liver microsomal fraction.
194 7-ethoxy or benzyloxyresorufin (0.1 mg ml⁻¹ in DMSO) were used as substrate (pH 7.5, 30°C). The
195 reaction was started by adding 10 μ l NADPH, and the increase in fluorescence was recorded for 4
196 minutes at $\lambda_{EX}=522$ nm/ $\lambda_{EM}=586$ nm. Activities were expressed as pmol min⁻¹ mg prot⁻¹ using a
197 standard curve generated with resorufin.

198 B(a)PMO activity was measured on 12 pools of microsomes (4 per site, 5 individuals each)
199 following the method of Kurelec et al. (1977). Assay conditions in the reaction mixture (final
200 volume 1.01 ml) were as follows: 110 mM Tris-HCl, 15 mM MgCl₂, 1.8 μ M NADPH and 100 μ l of
201 pooled microsomal fraction (pH 7.5, 30°C). B(a)P (2 mM) was used as substrate in a 30 min
202 reaction stopped with cool acetone. The amount of 3 OH-B(a)P produced was read at $\lambda_{EX}=396$
203 nm/ $\lambda_{EM}=522$ nm with H₂SO₄ 1 M and quinine sulphate 1 μ g/ml as standards. B(a)PMO activity was
204 determined in duplicates and compared to a blank treated with acetone prior to incubation; activity
205 was expressed in fluorescence units: min⁻¹ mg prot⁻¹.

206 BFCOD activity was measured according to Hasselberg et al. (2008). Reaction mixtures (200 μ l
207 total volume) contained: potassium phosphate buffer 0.2 M, 200 μ M BFC, bovine serum albumin
208 (1.6 mg/ml), NADPH 2 μ M and 10 μ l of microsomal fraction (pH 7.4, T 30°C). The reaction was
209 allowed to proceed for 30 minutes, and fluorescence was recorded using a VICTOR3 Multilabel
210 Counter (Wallac Sweden) at $\lambda_{EX}=410$ nm/ $\lambda_{EM}=538$ nm. Activities were expressed as pmol min⁻¹ mg
211 prot⁻¹ using a standard curve generated with 7-hydroxy-4-trifluoromethylcoumarin (HFC).

212 UDPGT activity was assayed according Collier et al. (2000): 15 μ l of 20 mM 5'-diphospho-
213 glucuronic acid was added to a reaction mixture containing 15 μ l of liver microsomal fraction and
214 120 μ l of 125 μ M 4-methyl-umbelliferon (4-MU) in 0.1 M Tris HCl containing 5 mM $MgCl_2$ and
215 0.05% BSA (pH 7.4) and incubated for 10 min at 37°C. Fluorescence was measured at $\lambda_{EX} = 355$
216 nm/ $\lambda_{EM} = 586$ nm. Activities were expressed as nmol min⁻¹ mg prot⁻¹ using a standard curve
217 generated with 4-MU.

218 GST activity was measured by the spectrophotometric method of Habig et al. (1974) modified for
219 microplate readers. 190 μ l CDNB 1 mM ($\epsilon=9.6$ mM cm⁻¹) dissolved in 0.1 M phosphate buffer (pH
220 7.42, T 18°C) and 10 μ l GSH 1.5 mM was added to 20 μ l diluted cytosolic fractions or
221 homogenizing buffer (reference). Absorbance was measured after 1 minute. Activities were
222 expressed as nmol min⁻¹ mg prot⁻¹.

223 Total proteins were measured according to Bradford (1976) using bovine serum albumin as
224 standard.

225

226 2.5 Statistical analysis

227 Comparisons between sampling sites were evaluated by ANOVA using the Bonferroni post-hoc test
228 except for B(a)PMO activity, for which the Mann-Whitney Wilcoxon rank sum non-parametric test
229 was used. Data was log-transformed to obtain more homogeneous values and correlations between
230 parameters were then determined by Pearson correlation coefficient (r). 0.05 was taken as
231 significance cut-off. Statistical analyses were performed with Statistica 7.1.

232

233 **3 Results**

234 3.1 PAHs

235 Concentration of the 16 PAHs measured in sediment from the three sites are reported in Table 2.
236 The highest concentrations were measured at HIS followed by MIS and REF, despite huge
237 variations in all three sites.

238 Similar levels of PAH were observed in fish fillets from specimens collected in HIS and MIS (Tab
239 2). Few inducers of CYP1A were detected in specimens from HIS, and the ranking was pyrene >
240 chrysene > benzo(a)anthracene, and likewise in MIS: pyrene > chrysene > benzo(k)fluoranthene. In
241 REF all PAHs were below the detection limit (Tab 2).

242

243 3.2 Biological responses

244 3.2.1 Molecular analysis

245 3.2.1.1 *cyp1a* and *cyp3a* sequences

246 A 534 bp product was obtained for *cyp1a* from liver of red mullet (GenBank accession GQ923895).
247 The sequence was more than 80% homologous with that of *D. labrax* (AJ251913.1 E value 1e-37),
248 *S. aurata* (E value 7e-27), *S. quinqueradiata* (AB09743.1 E value 5e-35), *L. saliens* (E value 7e-
249 27), *C. labrosus* (DQ438983.1 E value 2e-27), *P. platessa* (X73631.1 E value 1e-29) , *P. flesus*
250 (AJ130767.1 E value 6e-22) and *M. curema* (AY827103.1 E value 9e-27) *cyp1a*.

251 A 345 bp product was also obtained for *cyp3a* (GenBank accession GQ923894); it showed 70-79%
252 homology with *D. labrax* (DQ268535 E value 4 e-63), *M. salmoides* (*3a68* isoform (DQ786406.1 E
253 value 5e-56), *F. heteroclitus* (*3a56* isoform AY143428.1 E value 4 e-51, *3a30* isoform AF105068.2
254 E value 2e-48), *O. latipes* (AF105018.1 E value 3e-40) and *D. rerio* (*3a65* isoform AY452279.1 E
255 value 7e-35) *cyp3a*.

256

257 3.2.1.2 Real-time PCR

258 Significant up-regulation of *cyp1a* and *cyp3a* transcription was observed in red mullet from HIS
259 with respect to those from REF (REST: $p = 0.002$ and 0.021 respectively). *cyp1a* was also
260 significantly up-regulated in samples from MIS compared to those from REF ($p = 0.024$). Relative
261 induction of *abcc2* transcription was observed in mullet from HIS with respect to those from REF,
262 albeit not significant ($p = 0.051$) (Tab 3).

263 Liver microsomal enzyme activities such as EROD and B(a)PMO again showed significant
264 induction in specimens from HIS and MIS than from REF ($p < 0.05$) (Tab 3). A similar trend was
265 also observed for BFCOD with significantly higher activities in specimens from HIS than REF
266 ($p < 0.05$). No significant differences were observed for BROD activity ($p = 0.06$) or phase II
267 enzymes UDPGT and GST (Tab 4).

268 Correlations between transcriptional and biochemical parameters are shown in Table 5 as Pearson r
269 correlation coefficient and correlation significance. A marginally significant correlation of EROD,
270 BROD and BFCOD activities with *cyp1a* gene transcription was observed, whereas *cyp3a* did not
271 seem to correlate with BFCOD. No correlation was observed between *cyp1a* and the other two
272 genes, whereas a positive significant correlation was observed between *cyp3a* and *abcc2*. At the
273 biochemical level, strong correlations were observed among EROD, BROD and BFCOD activities.
274 UDPGT and GST did not show any correlation with other markers.

275

276 4 Discussion

277 Aim of the present study was to investigate the response to PAH contamination in liver of red
278 mullets from an oil-affected site by investigating modulation of phase I and III at transcriptional
279 level and phase I and II at post-transcriptional level.

280 PAH levels in sediments confirmed field exposure to contaminants of industrial origin, with higher
281 levels in HIS and MIS than in REF. HIS resulted *moderately contaminated* while MIS and REF
282 were *slightly contaminated* according to the worldwide sediment classification of Notar et al.
283 (2001). Observed PAHs proved to be of pyrolytic and petrogenic origin and known inducers of
284 CYP1A were detected, such as Benzo(a)pyrene and Dibenzo(a,h)anthracene (Lee and Anderson,
285 2005).

286 Regarding PAH concentrations in fillets, specimens from HIS had higher levels of contaminants
287 than red mullet from other Adriatic coastal areas (Corsi et al., 2002; Perugini et al., 2007), in line
288 with the hypothesis that HIS is characterized by high levels of PAHs contaminants, that in turn are
289 accumulated in fish tissues despite rapid biotransformation of these xenobiotics. On the other hand,
290 high molecular weight PAHs were not found in fillets from any of the three sites, despite their
291 presence in sediments, as a likely consequence of quick metabolization. Unfortunately, it was not
292 possible to assess PAHs biotransformation more systematically by investigating bile metabolites, as
293 the gall bladder was so small enabling the dissection from fish. Such measurement was nevertheless
294 retained as PAH content in mullet fillets is an important ecological and trophic indicator of transfer
295 to terminal consumers, including humans. The study of liver detoxifying responses at different
296 levels (transcriptional and post-transcriptional) in species from PAH-contaminated areas is therefore
297 important to understand the detoxifying capacity of the species and metabolism of PAHs under
298 natural conditions.

299 The significantly higher *cyp1a* transcription levels observed in liver of red mullet specimens from
300 HIS than in those from REF seem to confirm field exposure to PAHs known to be *cyp1a* inducers.
301 Similarly, EROD activity was significantly higher in liver of mullet from HIS than from REF,
302 confirming this induction. The extent of EROD induction was higher than the response at the *cyp1a*
303 gene level, observation that might nevertheless be biased by the relatively high transcript level in
304 mullet from REF. In line with this, EROD activity in fish from REF was high compared to activities
305 in fish from other moderately polluted areas though still well below those observed in fish from
306 harbour areas (Barcelona) (Porte et al., 2002; Corsi et al., 2002). This discrepancy in the extent of
307 induction has been previously reported for different species and type of contaminants. Quiròs and
308 collaborators (2007) underlined that the extent of *cyp1a* induction towards classical inducers (β -NF)
309 seemed species-specific and relatively high levels of *cyp1a* expression were also found in field
310 studies. Referring to field studies in oil-polluted marine areas, a low correlation between *cyp1a* gene
311 transcription and EROD activity was also reported by Kammann and collaborators (2008) in dab
312 (*Limanda limanda*) collected in the North and Baltic sea and in European flounder (*P. flesus*)
313 exposed to different contaminants (including PAHs) (George et al., 2004).

314 Several reasons can be hypothesized for the observed discrepancy in the extent of induction of
315 *cyp1a* gene transcription and EROD activity, such as different turnover rates and induction timing
316 between transcription and enzyme activity (Tukey and Johnson, 1990), different sensitivity towards
317 classical inducers at transcript level, mRNA processing, transport and stability, protein stability and
318 heme incorporation (Okey, 1990).

319 Regarding the other phase I activity, B(a)PMO response proved to be in line with that of EROD and
320 the level of PAH contamination of the sites. Compared to previous studies, measured activities were
321 similar to those observed in harbours and industrial settings and much higher than those measured
322 in relatively uncontaminated sites (Corsi et al., 2002).

323 *cyp3a* gene transcription levels were moderately up-regulated in specimens from HIS than REF,
324 whereas BFCOD activities showed significant positive correlations with dealkylating activities
325 (EROD and BROD) and *cyp1a* transcription. No correlation was otherwise observed between *cyp3a*
326 transcription and BFCOD activities. BFCOD is widely used as a measure of CYP3A activity,
327 although the substrate BFC is not strictly specific for CYP3A and is metabolized also by other
328 CYPs (Cheng et al. 2009; Renwick et al. 2000). Furthermore, fish show multiple *cyp3a* isoforms
329 with different expression patterns and distributions. Taken together our results suggest that
330 quantification of *cyp3a* transcription and BFCOD activity in liver of red mullet might not be
331 measurements of the same biological response. Nevertheless the good positive correlation between
332 BFCOD, *cyp1a* gene transcription and dealkylating activities underlines the need for further
333 investigation of genes encoding this activity and indicates BFCOD as a possible general marker of
334 CYP induction by PAHs, in this species. A CYP3A induction model using primary mullet
335 hepatocytes would be needed to establish whether CYP3A catalytic assays are reliable markers of
336 PAH contamination, as suggested in other species (Christen et al., 2009), and to investigate the
337 relationship between *cyp3a* and BFCOD.

338 Concerning phase II, our results seem in line with previous field studies that failed to observe any
339 alteration in GST and UDPGT activities in fish resident in PAH-contaminated sites (for a review
340 see van der Oost et al., 2003; Martinez-Gomez et al., 2009) although some evidence of inhibition of
341 GST activity has been reported in fish from PAH-contaminated sites (Bagnasco et al., 1991;
342 Tuvikene et al., 1999). The different trends observed for GST and UDPGT activities with respect to
343 other parameters is somehow unexpected as GST and UDPGT are actively involved in cell
344 detoxification and glutathione conjugates in particular are the preferential substrates of MRP2
345 (Leslie et al., 2005). A possible explanation could be found in the fact that the two enzymes may be
346 affected by additional factors beside the presence of PAHs. Furthermore, as both GST and UDPGT
347 have several isoforms with different functions and ligand preferences, the study of catalytic

348 activities alone might be a substantial limit to our complete understanding of the responses of these
349 enzymes.

350 In phase III, *abcc2* response appeared to be modulated to a lesser extent by PAH contamination
351 than *cyp1a* and *cyp3a*. A possible explanation could be that other ABC transporters besides MRP2
352 are involved in PAHs metabolites transport in red mullet liver as observed also in human Caco-2
353 cells (Lampen et al., 2004).

354 Regarding our aim to investigate detoxification pathways in relation to phase I, II and III, markers
355 of at least two phases (I, III) followed a similar trend of up-regulation in accordance with levels of
356 PAHs contamination, while markers of phase II appeared relatively stable. On an individual basis
357 significant correlations have been observed only between phase I markers, with the exception of
358 *cyp3a* that in turn correlates with *abcc2* (phase III). This is in line with observations in fish and
359 mammals where *cyp3a* and MRPs are often co-expressed (Bresolin et al., 2005; Xu et al., 2005).
360 Taken together these results suggest the occurrence of at least two groups of markers that are
361 coordinated but differently regulated in response to PAHs, possibly following distinct induction
362 pathways of response to environmental contamination.

363 To our knowledge, these are the first data on *cyp3a* and *abcc2* transcription in liver of a marine fish
364 species in the field. Together with CYP1A, both seem suitable candidates as markers of exposure,
365 although chemical inducers and regulation pathways remain to be studied in more details.
366 Furthermore, as CYP1A, CYP3A and MRP2 are involved in the metabolism and transport of
367 important endogenous compounds, including hormones (Hasselberg et al., 2008; Zaja et al., 2008),
368 this contaminant-induced modulation may impair important physiological functions and be
369 deleterious for the organism.

370

371 **5 Conclusions**

372 The present study provides the first data on responses of toxicologically important genes in a key
373 bioindicator species, the red mullet. CYP1A was confirmed as a sensitive marker of exposure to
374 PAHs in the field at transcription and post-transcription levels, though EROD proved to be a more
375 sensitive indicator of exposure under natural conditions. The low responsiveness of *cyp3a* and
376 *abcc2* to PAH contamination suggests that, although these genes could be potential markers of
377 exposure, further validation is needed. Our results indicate that BFCOD must be used with care as a
378 specific activity of *cyp3a* and might be better regarded as a general marker of CYP induction.
379 Transcription data on phase I and III in liver of red mullet is a first step towards understanding the
380 cell detoxification response at gene level for future pollution monitoring in the Mediterranean. As
381 biotransformation of PAHs is considered a prerequisite for carcinogenesis (van der Oost et al.,

382 1994), the cell response at mRNA level can provide an early warning of detrimental effects
383 occurring later at higher biological levels. Nevertheless, the low sensitivities observed for some of
384 these responses underline the need for extensive lab and field validation of mRNA-based
385 biomarkers in this species.

386

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391

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Table 1: Primers sequences, temperature and efficiency

Gene	Sense 5'-3'	Antisense 5'-3'	Temperature (°C)	Size bp	Accession number	Efficiency %
RT-PCR						
<i>cyp1a</i>	TTGTCAACGACCTGTTTGGGA	TATGTCCAGGAATGGTGTGG	55	534		
<i>cyp3a</i>	CTG TCC TTC ATC CCC TCC ACG	GGC CTG GTA CTC CAC AGG TTC	55	345		
qPCR						
<i>cyp1a</i>	CAACAAACTGGAAGGGGAGA	TGGATAATGATTGCCAAGAAGA	55	111	GQ923895	99.6
<i>cyp3a</i>	ATTAAATCCAACCGCGAGGGCA	AGGCCTTTACTCTGGGTTCCTT	55	113	GQ923894	98.2
<i>abcc2</i>	CTGGAAGTGCTGCCTGGAG	TGCAGGTAGACATCAGCGTCA	55	125	AY275434	105
<i>16S rRNA</i>	GGCCGGATCTTGTAGGTCAG	GCCCAAATGTTTTTGGTTGG	55	127	AF227679	109

Table 2: Concentrations of 16 most toxic PAHs indicated by EPA (ng/g) in sediments and fish fillets from the three sites. Results are mean of 25 distinct sediment samples collected at each site and 4 pools of fish fillets from each site. Range of $\Sigma 16$ PAHs is reported for sediments below detection limit (-).

	HIS		MIS		Ref	
	sediment	fillet	sediment	fillet	sediment	fillet
Naphtalene	47.64	9.49	1.73	8.70	5.13	-
Acenaphtylene	54.60	14.06	-	-	10.38	-
Acenaphtene	99.23	40.45	-	42.40	2.26	-
Fluorene	19.21	3.13	0.57	3.19	4.96	-
Phenanthrene	3.92	1.17	2.00	2.89	2.23	-
Anthracene	2.48	3.31	0.19	2.06	2.19	-
Fluoranthene	9.91	15.73	2.80	52.51	-	-
Pyrene	10.05	6.47	1.87	21.57	1.27	-
Benzo(g.h.i)pherylene	12.56	7.16	2.32	8.97	1.59	-
Benzo(a)anthracene	10.27	1.58	1.58	-	0.28	-
Chrysene	9.84	1.59	1.30	4.12	1.18	-
Benzo(b)fluoranthene	7.35	-	2.14	-	0.48	-
Benzo(k)fluoranthene	4.89	-	1.05	0.63	0.52	-
Benzo(a)pyrene	10.47	-	1.48	-	0.06	-
Dibenzo(a,h)anthracene	79.07	-	0.10	-	3.54	-
Indenopyrene	7.39	-	2.16	-	0.44	-
<i>Range $\Sigma 16$ PAHs</i>	<i>0.41 / 564.5</i>	<i>- / 124.28</i>	<i>- / 37.09</i>	<i>- / 78.77</i>	<i>- / 32.72</i>	<i>- / -</i>

Table 3 . Normalised relative transcription ratio of *cyp1a*, *cyp3a* and *abcc2* in red mullet from HIS and MIS obtained through REST Relative expression software 2008 V2.0.7. Data are expressed as relative fold induction with respect to the Reference site. *N* = 10 each site

	HIS			MIS		
	Relative fold induction	Std. Error range	p value	Relative fold induction	Std. Error range	p value
<i>cyp1a</i>	1.806	1.122 - 2.782	0.002	1.621	0.821 - 2.847	0.024
<i>cyp3a</i>	1.933	0.951 - 3.912	0.021	1.045	0.319 - 3.870	0.94
<i>abcc2</i>	1.825	0.852 - 3.855	0.051	0.856	0.336 - 2.194	0.693

Table 4. Hepatic microsomal and cytosolic enzyme activities of red mullet samples from the three sites. $N = 20$ each site

	HIS	MIS	Ref
EROD ^a	329.0 ± 38.86 *	241.8 ± 29.26 *	134.0 ± 14.55
BROD ^a	8.846 ± 0.90	7.368 ± 0.59	5.959 ± 0.43
B(a)PMO ^b	251.4 ± 38.19 *	140.5 ± 7.89 *	114.5 ± 7.48
BFCOD ^b	32.79 ± 6.56 *	16.89 ± 3.38	9.46 ± 3.31
GST ^c	76.80 ± 4.20	89.44 ± 4.36	83.64 ± 4.62
UDPGT ^c	2.534 ± 0.51	3.888 ± 0.26	2.913 ± 0.21

* significant difference ($p < 0.05$) with respect to the Reference site

^a pmol min⁻¹ mg prot⁻¹

^b UF min⁻¹ mg prot⁻¹

^c nmol min⁻¹ mg prot⁻¹

Table 5 .Correlations among *cyp1a*, *cyp3a* and *abcc2* gene transcription levels and BFCOD, EROD, BROD, GST and UDPGT enzymatic activities

		<i>cyp1a</i>	<i>cyp3a</i>	<i>abcc2</i>	BFCOD	EROD	BROD	GST	UDPGT
<i>cyp1a</i>	<i>r</i> coeff	1.00	0.2311	0.1198	0.4383	0.4348	0.4827	-0.1211	0.1270
	<i>p</i>		0.246	0.544	0.047	0.023	0.011	0.547	0.528
<i>cyp3a</i>	<i>r</i> coeff		1.00	0.6734	0.0502	-0.0279	-0.0141	0.0959	-0.1910
	<i>p</i>			0.000	0.829	0.892	0.946	0.641	0.350
<i>abcc2</i>	<i>r</i> coeff			1.00	0.0532	0.0117	-0.0343	0.2477	-0.3217
	<i>p</i>				0.819	0.954	0.865	0.213	0.102
BFCOD	<i>r</i> coeff				1.00	0.8652	0.8650	-0.2586	0.4262
	<i>p</i>					0.000	0.000	0.258	0.054
EROD	<i>r</i> coeff					1.00	0.8170	-0.950	0.2598
	<i>p</i>						0.000	0.644	0.191
BROD	<i>r</i> coeff						1.00	-0.0827	0.2915
	<i>p</i>							0.688	0.140
GST	<i>r</i> coeff							1.00	0.1829
	<i>p</i>								0.371
UDPGT	<i>r</i> coeff								1.00

r = Pearson correlation coefficient

p = significance level ($p < 0.05$ is considered significant)