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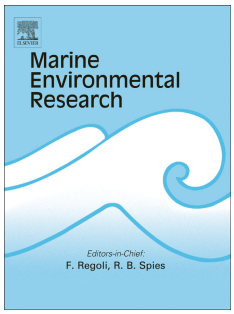

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Transcriptional and post-transcriptional response of drug-metabolizing enzymes to PAHs contamination in red mullet (*Mullus barbatus*, Linnaeus, 1758): a field study

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Abstract

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

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

1 Introduction

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

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

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

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

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

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

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

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

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

2 Methods

2.1 Fish and sediment sampling

Specimens of red mullet were captured in November 2007 in the north-eastern Adriatic off a stretch of coast characterized by high human impact, extending from 43.62816°N, 13.44080°E to 43.68213°N, 13.31534°E. Three sites with different degrees of PAHs contamination were considered: one site was directly off an oil refinery (high-impact site HIS) (43.64152°N, 13.40688°E). The refinery occupies an area of 70 ha, processes 3.9 million tons of crude oil per year and produces 2 million MWh/y. Storage capacity (about 1,500,000 m^3) is among the largest in Europe. All the crude oil arrives by sea and refinery products leave by sea (30%) and land (70%). The many transport and processing operations lead to frequent accidental spills (the most recent of fuel oil in April 2007). The other two sites were at increasing distances from HIS, one defined as moderate impact (MIS) affected by urban run-off (43.66269°N, 13.34753°E) and the other as reference (REF) (43.67660°N, 13.33383°E). The area normally has slow sea currents (2.5 m/sec) flowing NW-SE direction, i.e. from the REF to the MIS and HIS sites. The sites were in a 7-Km stretch of sea about 1300 m from the shore. Trawling was carried out for 900 m at depths between 20-30 m.

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

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

2.2 PAH analysis

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

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

2.3 Molecular analysis

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

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

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

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

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

2.4 Biochemical assays

Liver cytosolic and microsomal fractions were obtained as described by Corsi et al. (2003). Microsomal EROD and BROD activities were measured according to the fluorimetric methods of Burke and Mayer (1974). Assay conditions in the reaction mixture (final volume 2.25 ml) were as follows: 50 mM Tris-HCl, 25 mM $MgCl_2$, 125 µM NADPH and 50 µl of liver microsomal fraction. 7-ethoxy or benzyloxyresorufin (0.1 mg ml^{-1} in DMSO) were used as substrate (pH 7.5, 30°C). The reaction was started by adding 10 µl NADPH, and the increase in fluorescence was recorded for 4 minutes at λ_{EX}=522 nm/λ_{EM}=586 nm. Activities were expressed as pmol min^{-1} mg $prot^{-1}$ using a standard curve generated with resorufin.

B(a)PMO activity was measured on 12 pools of microsomes (4 per site, 5 individuals each) following the method of Kurelec et al. (1977). Assay conditions in the reaction mixture (final volume 1.01 ml) were as follows: 110 mM Tris-HCl, 15 mM $MgCl_2$, 1.8 µM NADPH and 100 µl of pooled microsomal fraction (pH 7.5, 30°C). B(a)P (2 mM) was used as substrate in a 30 min reaction stopped with cool acetone. The amount of 3 OH-B(a)P produced was read at λ_{EX}=396 nm/λ_{EM}=522 nm with H_2SO_4 1 M and quinine sulphate 1 µg/ml as standards. B(a)PMO activity was determined in duplicates and compared to a blank treated with acetone prior to incubation; activity was expressed in fluorescence units: min^{-1} mg $prot^{-1}$.

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

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

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

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

2.5 Statistical analysis

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

3 Results

3.1 PAHs

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

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

3.2 Biological responses

3.2.1 Molecular analysis

3.2.1.1 cyp1a and cyp3a sequences

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

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

3.2.1.2 Real-time PCR

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

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

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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

5 Conclusions

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

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

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References

Aas, E., Baussant, T. Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquatic Toxicology 51, 241–258.

Ausili, A., Gabellini, M., Cammarata, G., Fattorini, D., Benedetti, M., Pisanelli, B., Gorbi, S., Regoli, F., 2008. Ecotoxicological and human health risk in a petrochemical district of southern Italy. Marine Environmental Research 66, 215-217.

Bagnasco, M., Camoirano, A., De Flora, S., Melodia, F., Arillo, A., 1991. Enhanced liver metabolism of mutagens and carcinogens in fish living in polluted seawater. Mutation Research 262, 129-137.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquatic Toxicology 48, 357–389.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Bresolin, T., de Freitas Rebelo, M., Bainy, A.C.D., 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. Comparative Biochemistry and Physiology C 140, 403-407.

Burgeot, T., Bocquene, G., Pingray, G., Godefroy, D., Legrand, J., Dimeet, J., Marco, F., Vincent, F., Henocque, Y., Jennaret, H.O., Galgani, F., 1994. Monitoring biological effects of contamination in marine fish along French coasts by measurement of ethoxiresorufin-O-deethylase activity. Ecotoxicology and Environmental Safety 29, 131-147.

Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorimetric assay of microsomal O-dealkylation which is preferentially induced by 3 methylcholantrene. Drug Metabolism and Disposition 2, 583-588.

415 Cheng, Q., Christal, D.S., Guengerinch, F.P., 2009. High-throughput fluorescence assay of
416 cytochrome P450 3A4. Nature Protocols 4(9), 1258-1259.

417 Christen, V., Oggier, D.M., Fent K., 2009. A Microtiter-Plate Based Cytochrome P4503A Activity
418 Assay in Fish Cell Lines. Environmental Toxicology and Chemistry 26:1.

419 Collier, A.C., Tingle, M.D., Keelan, J.A., Paxton, J.W., Mitchell, M.D., 2000. A highly sensitive
420 fluorescent microplate method for the determination of UDP-glucoronosyl transferase activity in
421 tissues and placental cell lines. Drug Metabolism and Disposition 28, 1184-1186.

422 Corsi, I., Mariottini, M, Menchi, V., Sensini, C., Balocchi, C., Focardi, S., 2002. Monitoring a
423 marine coastal area: use of *Mytilus galloprovincialis* and *Mullus barbatus* as bioindicators. Marine
424 Ecology 23, 138-153.

425 Corsi, I., Mariottini, M., Sensini, C., Lancini, L., Focardi, S., 2003. Cytochrome P450,
426 acetylcholinesterase and gonadal histology for evaluating contaminant exposure levels in fishes
427 from a highly eutrophic brackish ecosystem: Orbetello Lagoon, Italy. Marine Pollution Bulletin 46,
428 203-212.

429 George, S., Gubbins, M., MacIntosh, A., Reynolds, W., Sabine, V., Scott, A., Thain, J., 2004. A
430 comparison of pollutant biomarker responses with transcriptional responses in European flounders
431 (*Platicthys flesus*) subjected to estuarine pollution. Marine Environmental Research 58, 571–575.

432 Goksøyr, A., Förlin, L., 1992. The cytochrome P-450 system in fish, aquatic toxicology and
433 environmental monitoring. Aquatic Toxicology 22, 287–312.

434 Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step
435 in mercapturic acid formation. Journal of Biological Chemistry 249, 7130-7139.

436 Hahn, M.E., 1998. The aryl hydrocarbon receptor: a comparative perspective. Comparative
437 Biochemistry and Physiology C 121, 23-53.

438 Harmelin-Vivien, M., Cossa, D., Crochet, S., Bănaru, D., Letourneur, Y., Mellon-Duval, C., 2009.
439 Difference of mercury bioaccumulation in red mullets from the north-western Mediterranean and
440 Black seas. Marie Pollution Bulletin 58, 679–685.

441 Hasselberg, L., Westerberg, S., Wassmur, B., Celander, M.C., 2008. Ketoconazole, an antifungal
442 imidazole, increases the sensitivity of rainbow trout to 17α-ethynylestradiol exposure. Aquatic
443 Toxicology 86, 256-264.

444 IARC, 1989. Diesel and Gasoline Engine Exhausts and some Nitroarenes. IARC Monographs on
445 the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 46, Lyon, International Agency
446 for Research of Cancer, France.

447 Insausti, D., Carrasson, M., Mainou, F., Cartes, J.E., Solè, M., 2009. Biliary fluorescent aromatic
448 compounds (FACs) measured by fixed wavelength fluorescence (FF) in several marine fish species
449 from the NW Mediterranean. Marine Pollution Bulletin 58, 1635-1642.
450 James, M.O., Zhen, L., Rowland-Faux, L., Celander, M., 2005. Properties and regional expression
451 of a CYP3A-like protein in channel catfish intestine. Aquatic Toxicology 72, 361-371.
452 Jewett, S.C., Dean, T.A., Woodin, B.R., Hoberg, M.H., Stegeman, J.J., 2002. Exposure to
453 hydrocarbons 10 years after the Exxon Valdez oil spill: evidence from cytochrome P4501A
454 expression and biliary FACs in near shore demersal fishes. Marine Environmental Research 54, 21–
455 48.
456 Kamman, U., Lang, T., Berkau, A-J., Klempt, M., 2008. Biological effect monitoring in dab
457 (*Limanda limanda*) using gene transcript of CYP1A1 or EROD-a comparison. Environmental
458 Sciences and Pollution Research 15, 600-605.
459 Kurelec, B., Britvic, S., Rijavec, M., Muller, W.E.G., Zahn, R.K., 1977. Benzo(a)pyrene
460 monooxigenase induction in marine fish. Molecular response to oil pollution. Marine Biology
461 44,211-216.
462 Lampen, A., Ebert, B., Stumkat, L., Jacob, J., Seidel, A., 2004. Induction of gene expression of
463 xenobiotic metabolism enzymes and ABC-transport proteins by PAH and reconstituted PAH
464 mixture in human Caco-2 cells. Biochimica and Biophysica Acta 1681, 38-46.
465 Lee, R.F., Anderson J.W., 2005. Significance of cytochrome P450 system responses and levels of
466 bile fluorescent aromatic compounds in marine wildlife following oil spills. Marine Pollution
467 Bulletin 50, 705-723.
468 Leslie, E.M., Deeley, R.G., Cole, S.P., 2005. Multidrug resistance proteins: role of P-glycoprotein,
469 MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicology and Applied Pharmacology 204,
470 216-237.
471 Li, D., Yang, X-L., Zhang S-J., lin, M., Yu, W-J., Hu, K., 2008. effects of mammalian CYP3A
472 inducers on CYP3A-related enzyme activities in grass carp (*Ctenopharyngodon idellus*): possible
473 implications for the establishment of a fish CYP3A induction model. Comparative Biochemistry
474 and Physiology C 147, 17-29.
475 Martin-Skilton, R., Thibaut, R., Porte, C., 2006a. Endocrine alteration in juvenile cod and turbot
476 exposed to dispersed crude oil and alkylphenols, Aquatic Toxicology 78S, S57–S64.
477 Martin-Skilton, R., Lavado, R., Thibaut, R., Minier, C., Porte, C., 2006b. Evidence of endocrine
478 alteration in the red mullet, *Mullus barbatus* from the NW Mediterranean. Environmental Pollution
479 141, 60-68.

480 Martínez-Gómez, C., Fernández, B., Valdés, J., Campillo, J.A., Benedicto, J., Sánchez, F.,
481 Vethaak, A.D. 2009. Evaluation of three-year monitoring with biomarkers in fish following the
482 Prestige oil spill (N Spain). Chemosphere 74, 613-620.
483 Mathieu, A., Lemaire, P., Carriere, S., Drai, P., Giudicelli, J., Lafaurie, M., 1991. Seasonal and sex-
484 linked variations in hepatic and extrahepatic biotransformation activities in Striped mullet (*Mullus*
485 *barbatus*). Ecotoxicology and Environmental Safety 22, 45-57.
486 Miller, D.S., Shaw, J.R., Stanton, C.R., Barnaby, R., Karlson, K.H., Hamilton, J.W., Stanton, B.A.,
487 2007. MRP2 and Acquired Tolerance to Inorganic Arsenic in the Kidney of Killifish (*Fundulus*
488 *heteroclitus*). Toxicological Sciences 97, 103–110.
489 Notar, M., Leskovšek, H., Faganeli, J., 2001. Composition, Distribution and Sources of Polycyclic
490 Aromatic Hydrocarbons in Sediments of the Gulf of Trieste, Northern Adriatic Sea. Marine
491 Pollution Bulletin 42, 36-44.
492 Okey A.B., 1990. Enzyme induction in the cytochrome P-450 system. Pharmacology &
493 Therapeutics 45, 241–298.
494 Paetzold, C. S., Ross, N.W., Richards, R.C., Jones, M., Hellou, J., Bard, S.M. 2009. Up-regulation
495 of hepatic ABCC2, ABCG2, CYP1A1 and GST in multixenobiotic-resistant killifish (*Fundulus*
496 *heteroclitus*) from the Sydney Tar Ponds, Nova Scotia, Canada. Marine Environmental Research
497 68, 37-47.
498 Perugini, M., Visciano, P., Giammarino, A., Manera, M., Di Nardo, W., Amorena, M., Polycyclic
499 aromatic hydrocarbons in marine organisms fro the Adriatic Sea, Italy. Chemosphere 66, 1904-
500 1910.
501 Pfaffl, M., 2001. A new mathematical model for relative quantification in real-time RT-PCR.
502 Nucleic Acids Research 29, 2002-2007.
503 Porte, C., Escartin, E., Gracia de la Parra, L.M., Biosca, X., Albaiges, J., 2002. Assessment of
504 coastal pollution by combined determination of chemical and biochemical markers in Mullus
505 Barbatus. Marine Ecology Progress Series 235, 205-216.
506 Quiròs, L., Piña, B., Sole´, M., Blasco, J., Ángel Lòpez, M., Riva, M.C., Barcelo´, D., Raldùa, D.,
507 2007. Environmental monitoring by gene expression biomarkers in *Barbus graellsii*: Laboratory
508 and field studies. Chemosphere 67, 1144–1154.
509 Regoli, F., Pellegrini, D., Winston, G.W., Gorbi, S., Giuliani, S., Virno-Lamberti, C., Bompadre, S.,
510 2002. Application of biomarkers for assessing the biological impact of dredged materials in the
511 Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress
512 in the red mullet (Mullus barbatus). Marine Pollution Bulletin 44, 912-922.

513 Renwick, A.B., Surry, D., Price, R.J., Lake, B.G., Evans, D.C., 2000. Metabolism of 7-benzyloxy-
514 4-trifluoromethyl-coumarin by human hepatic cytochrome P450 isoforms. Xenobiotica 30, 955-
515 969.
516 Sauerborn, R., Stupin Polancec, D., Zaja, R., Smital, T., 2004. Identification of the multidrug
517 resistance-associated protein (mrp) related gene in red mullet (*Mullus barbatus*) Marine
518 Environmental Research 58, 199-204.
519 Shailaja, M.S., D'Silva C., 2003. Evaluation of impact of PAH on a tropical fish, *Oreochromis*
520 *mossambicus* using multiple biomarkers. Chemosphere 53, 835–841.
521 Smith, E.M., Wilson, J.Y., 2010. Assessment of cytochrome P450 fluorimetric substrates with
522 rainbow trout and killifish exposed to dexamethasone, pregnenolone-16α-carbonitrile, rifampicin,
523 and β-naphtoflavone. Aquat. Toxicol. doi:10.1016/j.aquatox.2010.01.005.
524 Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenases: current
525 perspectives on forms, functions and regulation of cytochrome P450 in aquatic species. In: Malins,
526 D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology: Molecular, Biochemical, and Cellular
527 Perspectives. Lewis Publisher Inc., Boca Raton, FL, pp. 87-204.
528 Tukey, R.H., Johnson, E.F., 1990. Molecular aspects of drug metabolizing enzymes. In Pratt, W.,
529 Taylor, P., (Eds), Principles of drug action. Churcill Livingstone, New York, pp 423-468.
530 Tuvikene, A., Huuskonen, S., Koponen, K., Ritola, O., Mauer, U., Lindström-Seppa, P., 1999. Oil
531 shale processing as a source of aquatic pollution: monitoring of the biologic effects in caged and
532 feral freshwater fish. Environmental Health Perspectives 107, 745-752.
533 UNEP/RAMOGE, 1999. Manual on the biomarkers recommended for the MED POL biomonitoring
534 programme. UNEP, Athens.
535 US-EPA, 1996. US EPA method 3545B. Pressurized Fluid Extraction (PFE), Office of Water,
536 Washington, D.C.
537 Vaccaro, E., Salvetti, A., Del Carratore, R., Nencioni, S., Longo, V., Gervasi, P.G., 2007. Cloning,
538 tissue expression, and inducibility of CYP3A79 from Sea Bass (*Dicentrarchus labrax*). Journal of
539 Biochemical and Molecular Toxicology 21, 32-40.
540 van der Oost, R., van Schooten, F.J., Ariese, F., Heida, H., Vermeulen, N.P.E., 1994.
541 Bioaccumulation, Biotransformation and DNA binding of PAHs in feral eel (*Anguilla anguilla*)
542 exposed to polluted sediments: a field survey. Environmental Toxicology and Chemistry 13, 859-
543 870.
544 van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in
545 environmental risk assessment: a review. Environental Toxicology and Pharmacology 13, 57-149.

546 Venturini, N., Muniz, P., Bícego, M.C., Martins, C.C., Tommasi, L.Z., 2008. Petroleum
547 contamination impact on macrobenthic communities under the influence of an oil refinery:
548 Integrating chemical and biological multivariate data. Estuarine, Coastal and Shelf Science 78, 457-
549 467.
550 Xu, C.C., Yong-Tao, L., Kong, A.N.T., 2005. Induction of Phase I, II, III Drug
551 Meatabolism/Transport by Xenobiotics. Archives of Pharmacology Research 28, 249-268.
552 Zaja, R., Munić, V., Sauerborn Klobučar, R., Ambriović-Ristov, A., Smital, T., 2008. Cloning and
553 molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow
554 trout (Oncorhynchus mykiss) hepatocytes. Aquatic Toxicology 90, 322-332.
555 Zorita, I., Ortiz-Zarragoitia, M., Apraiz, I., Cancio, I., Orbea, A., Soto, M., Marigòmez, I.,
556 Cajaraville, M.P., 2008. Assessment of biological effects of environmental pollution along the NW
557 Mediterranean Sea using red mullets as sentinel organisms. Environmental Pollution 153, 157-168.
558 Zucchi, S. 2009. ABC transporters in the Antarctic Emerald rock cod *Trematomus bernacchii*.
559 Doctoral thesis 142 pp.
560

Table 1: Primers sequences, temperature and efficiency

Gene	Sense 5'-3'	Antisense 5'-3'	Temperature (°C)	Size bp	Accession number	Efficiency %
	RT-PCR					
cyp1a	TTGTCAACGACCTGTTTGGA	TATGTCCAGGAATGGTGTGG	55	534		
cyp3a	CTG TCC TTC ATC CCC TCC ACG	GGC CTG GTA CTC CAC AGG TTC	55	345		
	qPCR					
cyp1a	CAACAAACTGGAAGGGGAGA	TGGATAATGATTGCCAAGAAGA	55	111	GQ923895	99.6
cyp3a	ATTAAATCCAACCGCGAGGGCA	AGGCCTTTACTCTGGGTTCCTT	55	113	GQ923894	98.2
abcc2	CTGGAAGTGCTGCCTGGAG	TGCAGGTAGACATCAGCGTCA	55	125	AY275434	105
16S rRNA	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 Σ16 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	-
Range Σ16 PAHs	*0.41 / 564.5*	*- / 124.28*	*- / 37.09*	*- / 78.77*	*- / 32.72*	*- / -*

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
cyp1a	1.806	1.122 - 2.782	0.002	1.621	0.821 - 2.847	0.024
cyp3a	1.933	0.951 - 3.912	0.021	1.045	0.319 - 3.870	0.94
abcc2	1.825	0.852 - 3.855	0.051	0.856	0.336 - 2.194	0.693

Table 4. Hepatic microsomal and citosolic 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

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

r = Pearson correlation coefficient
p = significance level ($p<0.05$ is considered significant)