Biliary PAH and alkylphenol metabolites, biomarker enzyme activities and gene expression levels in the deep-sea fish *Alepocephalus rostratus*

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Abstract

Biliary polycyclic aromatic hydrocarbon (PAH) and alkylphenol (AP) metabolites, hepatic gene expression and corresponding enzyme activities were determined in the deep-sea fish *Alepocephalus rostratus* from two sites within the Mediterranean. Biliary metabolites included the hydroxylated PAH metabolites (OH-PAHs) 1-naphthol, 2-naphthol, 9-fluorenone, 9-phenanthrene and 1-pyrene and the APs 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP). Five biomarker genes, namely cytochrome P450 1A (CYP1A), vitellogenin (Vtg), catalase (CAT), Cu/Zn-superoxide-dismutase (Cu/Zn-SOD) and glutathione reductase (GR) were quantified using qRT-PCR. Moreover, corresponding enzyme activities (EROD, CAT, SOD and GR) were also determined. The Σ OH-PAHs detected ranged from 21.1-300.3 ng/g bile and were mainly comprised of 1-naphthol. Both, NP and OP metabolites were detected in all samples with concentrations ranging from 17.4-107.2 ng/g bile and 4.9-17.3 ng/g bile, respectively, and levels were significantly higher in samples from the western Mediterranean (WM) compared to those from the Catalan slope (CS). Accordingly, gene expression was significantly induced in male fish from the WM, however, these results were not reflected in enzyme activity levels. In particular, males caught at 2000 m in the WM exhibited 35-times higher Vtg levels compared to those from the CS, suggesting that endocrine-disrupting effects may potentially be occurring in such remote environments as the deep-sea.

Keywords: gene expression, biomarkers; vitellogenin (Vtg); alkylphenols (AP); polycyclic aromatic hydrocarbons (PAH); bile metabolites, deep-sea fish; Mediterranean

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1 **Introduction**

2 For a long time the deep-sea was considered a relatively pristine environment due to its
3 remoteness from anthropogenic pollution sources. However, over the last decades
4 research has shown that the deep-sea may actually act as a sink for persistent
5 contaminants that enter the marine environment ¹⁻⁵. Among these contaminants,
6 polycyclic aromatic hydrocarbons (PAHs) have been shown to be transported to deeper
7 waters by sinking particles ⁶, where they accumulate in deep-sea organisms ⁷⁻⁸. PAHs
8 are a unique class of ubiquitous persistent pollutants, originating from natural and
9 anthropogenic sources such as incomplete combustion processes (pyrogenic) and the
10 release of crude oil and petroleum products (petrogenic).

11 Alkylphenols (APs) represent a class of nonionic surfactants that are extensively used in
12 detergents, pesticide formulations, fuel, lubricants and many other industrial products,
13 with octylphenol and nonylphenol ethoxylates being the most common surfactants
14 applied. APs are released via municipal wastewater or directly discharged into the
15 environment, where they degrade to more persistent and toxic shorter-chain compounds
16 such as nonylphenol (NP) and octylphenol (OP) ⁹. Many studies have found AP
17 metabolites in a wide range of environmental samples ⁹⁻¹⁰ and there has been growing
18 concern on the toxicity of these compounds due to their ability to mimic natural
19 hormones by interfering with the estrogen-receptor ¹¹. However, little is known about
20 the environmental fate of APs in the marine environment and only few studies have
21 addressed the issue of AP exposure in marine organisms ¹⁰. Moreover, the presence of
22 APs has thus far not been investigated in deep-sea biota, although a study by Kannan et
23 al. ¹² detected a peak of NP in Japanese waters at 1000 m depth.

24 Once taken up by an organism, xenobiotic compounds including PAHs and APs
25 undergo a series of biotransformation processes, before being excreted through the bile
26 as hydroxylated and/or conjugated metabolites. Thus, biliary metabolites of these
27 contaminants can be used as exposure markers in fish ¹³. Furthermore, the activation of
28 enzymatic biotransformation systems and subsequent effects on, for instance,
29 antioxidant responses can serve as ecotoxicological tools to assess the exposure to these
30 pollutants ¹⁴.

31 The cytochrome P450 (CYP) system plays a key role in the Phase I metabolism of many

1 xenobiotics. In fish, CYP1A induction has been used as a robust biomarker to assess the
2 exposure to organic contaminants such as PAHs and polychlorinated biphenyls (PCBs)
3 ¹⁵⁻¹⁶. Moreover, the exposure to organic pollutants can also cause oxidative stress due to
4 the generation of reactive oxygen species (ROS) ¹⁷. Increased oxidative stress can lead
5 to the induction of antioxidant enzymes such as catalase (CAT), superoxide-dismutase
6 (SOD) and glutathione reductase (GR) as a protective mechanism against these
7 oxidative radicals ¹⁷⁻¹⁸. The induction of vitellogenin (Vtg) has been used as a marker to
8 assess the exposure of aquatic organisms to xenoestrogens ¹⁹. Vitellogenins are the
9 precursors of the egg-yolk proteins vitellins and their production is under the control of
10 the estrogen receptor in vertebrates. Vtg levels generally increase during sexual
11 maturation of females, but are usually undetectable in males ²⁰. However, exposure to
12 estrogenic compounds including APs, can lead to the expression of Vtg genes in males,
13 as has been observed in laboratory as well as field studies on freshwater and marine fish
14 ²¹⁻²⁵.

15 The objectives of the present study were to assess the PAH and AP exposure in the
16 deep-sea fish *Alepocephalus rostratus*, together with the activity of hepatic enzymatic
17 activities (i.e. EROD, CAT, GR and SOD) that are widely used as biomarkers.
18 Additionally, gene expression profiles of CYP1A, CAT, GR, Cu/Zn-SOD and Vtg were
19 determined using qRT-PCR. Particular emphasis was placed on the detection of Vtg
20 mRNA levels as a marker of estrogenic exposure. The present work is the first study to
21 assess biliary AP metabolites and to apply quantitative gene expression biomarker
22 techniques to deep-sea fish.

23 24 **Experimental section**

25 **Sample collection**

26 Specimens of *A. rostratus* were collected from the Catalan slope (CS) during February
27 2009 (bottom temperature 13.1 °C) and the western Mediterranean (WM) during May
28 2009 (bottom temperature 13.1 °C) (Figure 1), using an OTMS otter trawl ²⁶ at 1500 m
29 depth on the CS and 1200 m and 2000 m in the WM. Sample details are provided in
30 Table 1. *A. rostratus* is a demersal fish species with a relatively wide depth distribution

(300-2300 m), with an abundance peak between 1000 m and 1500 m depth. It is mainly found in the western Mediterranean, where it represents one of the most abundant megafauna species and mainly feeds on gelatinous macroplankton ²⁸. Previous studies have found mature individuals of *A. rostratus* all year round, although a clear peak in spawning activity was observed during summer and autumn ²⁹. The maturity stage of the sampled individuals is also presented in Table 1, based on a five-point scale by Brown-Peterson et al. ²⁷ (1: immature; 2: developing-regenerating; 3: spawning capable; 4: actively spawning; 5: regressing). Onboard, the bile and liver were dissected and immediately frozen at -20 °C and -80 °C, respectively, until further treatment.

Analysis of hydroxylated PAHs and alkylphenol metabolites in bile

Bile samples were analyzed as described in Fernandes et al. ³⁰. Briefly, 100 mg of bile was incubated for 1 h at 40 °C in 1 mL 0.4 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 U of β-glucuronidase and 50 U sulfatase. The bile of two or more individuals was pooled if the amount of sample was <100 mg. Before analysis, hydrolyzed samples were derivatized by the addition of 100 μL bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and samples were left to stand overnight at room temperature. Analyses were carried out by gas-chromatography-mass spectrometry (GC-MS-EI). The derivatized ions used for the quantification of hydroxylated-PAHs were: m/z 216 and 201 for 1-naphthol; m/z 254 and 165 for 9-fluorenone; m/z 266 and 251 for 9-phenanthrene; and m/z 290 for 1-pyrene. The ions used for monitoring and quantification of APs were: m/z 207 and 193 for 4-nonylphenol (NP) and m/z 207 for 4-tert-octylphenol (OP). Concentrations are expressed as ng/g bile. Detection limits were calculated as signal to noise ratio of 3:1 and were in the order of 1-5 pg for OH-PAHs and 10 pg for NP and 7 pg OP. Sample sizes were OS (n = 12) and BC (n = 9).

Enzymatic activities

A portion of liver (approx 0.5 g) was homogenized 1:4 (w:v) in a 100 mM phosphate buffer pH 7.4 containing 150 mM KCl, 1 mM dithiotreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 g for 30 min and the supernatant (S9) was used for biochemical analyses.

All assays were carried out in triplicate at 25 °C, except if specified differently, in a 96-well format using a TECAN™ Infinite M200 microplate reader as described in ³¹. Briefly, CAT activity was measured as absorbance decrease at 240 nm for 1 min using 50 mM H₂O₂ as substrate ($\epsilon = 40 \text{ mol}^{-1} \cdot \text{cm}^{-1}$) and a 100 mM phosphate buffer pH 6.5 ³². GR activity was measured as decrease in absorbance at 340 nm for 3 min using 0.09 mM nicotinamide adenine dinucleotide phosphate (NADPH) ($\epsilon = 6.22 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$) and 0.9 mM oxidized glutathione (GSSG) as substrate ³³. SOD activity was determined as inhibition of the reduction of cytochrome c at 550 nm, following the method for microplate format as described in Kopecka-Pilarczyk and Correia ³⁴. The reaction contained 50 μM hypoxanthine, 1.8 mUml^{-1} xanthine oxidase and 10 μM cytochrome c. EROD activity was measured kinetically at 30 °C based on the procedure by Burke and Mayer ³⁵, using 7-ethoxyresorufin (3 μM) as substrate and 0.2 mM NADPH. Protein content was determined according the method by Bradford ³⁶, using bovine serum albumin as standard (BSA 0.1–1 mg/ml).

Quantitative real-time PCR (qPCR)

A standard curve containing six dilution series of pooled cDNA samples (40 ng–1.25 ng) was used to ensure an optimized assay for each primer pair (efficiency 100 % $\pm$ 5%). The qPCR was performed in an iQ5 Thermal cycler (Bio-Rad) on a 96-well qPCR plate (4titude). The amplification reaction was performed in duplicates using the fluorescent dye iQ™ SYBR®Green Supermix (Bio-Rad) with 20 ng sample cDNA resulting in a final reaction volume of 20 μl /well. Respective primer had a ‘non template control’ (NTC) in the amplification reaction to control for primer and mastermix contamination. PCR was achieved by a 1 min activation step at 95 °C, followed by 40 cycles of a 20 s denaturing step at 95 °C, a 20 s primer annealing step at 60 °C and a 30 s elongation step at 72 °C. A melting curve was performed at each run in order to confirm specific amplification of each sample. An accepted Ct-value of NTC and NoRT samples was set to be either non-detectable or >32 cycles. Housekeeping gene expression was used to normalize quantitative PCR cycle threshold (Ct) data ³⁷. However, previous studies have shown that the expression of commonly used reference genes can also be altered by toxicological processes ^{38–39}. Therefore, in the present work the geometric mean of three reference genes, namely β -actin, elongation factor 1a (EF1a) and ribosomal RNA 12S, was used to minimize the potential

1 variability of housekeeping gene expression. The calculated geometric mean of
2 reference gene values did not differ between sites ($p = 0.9$).

3 **Statistical analysis**

4 Data were tested for normality (Shapiro-Wilk's test) and homogeneous variance
5 (Levene's test). For bile metabolites, data were found to satisfy both assumptions and
6 site comparisons were performed using the parametric Student's t-test. For biomarker
7 and gene expression data, the assumptions of normality and homogeneous variance
8 were not satisfied and differences between sites were analyzed using the Mann-Whitney
9 U test. Correlations were determined using the Spearman's rank correlation (ρ) to
10 account for non-linear relationships. Differences at the 5% significance level were
11 considered significant.

13 **Results and discussion**

14 **Bile metabolites**

15 The levels of biliary Σ OH-PAHs detected in *A. rostratus* varied between 21.1 and 300.3
16 ng/g bile (Table 2). Of the five PAH metabolites analyzed, 1-naphthol represented on
17 average 90 % of Σ OH-PAHs, with levels ranging from 15.5 to 287.3 ng/g bile, while the
18 concentrations for 2-naphthol, 9-fluorenel, 9-phenantrol and 1-pyrenol were relatively
19 low or below detection limits (Table 2). Although pyrenol has been regarded as the key
20 PAH metabolite in fish ⁴⁰, this compound was almost not detectable in the present
21 study. The high abundance of naphthol indicates a petrogenic origin of PAH
22 contamination, similar to results found in two fish species sampled along the northern
23 Iberian shelf off Galicia ³⁰, but in contrast to previous results on PAH metabolites in *A.*
24 *rostratus* from the NW Mediterranean, where pyrenol was the dominant biliary
25 metabolite ⁸. Σ OH-PAHs levels were slightly higher than those measured for fish from
26 the northern Iberian shelf, Atlantic Sea ³⁰, but similar to previous results reported for *A.*
27 *rostratus* from the NW Mediterranean ⁸.

28 To our knowledge, the present study is the first to determine AP metabolites in deep-sea
29 fish. NP and OP were present in all bile samples, with concentrations of OP

1 approximately 5 times lower than NP (Table 2). Only a limited number of field studies
2 have measured AP metabolites in fish bile and the levels of NP and OP detected in the
3 present study are within the same range of concentrations found in the previously-
4 mentioned study from the northern Iberian shelf ³⁰, but lower than the concentrations
5 observed in bile of the red mullet (*Mullus barbatus*) from the NW Mediterranean ⁴¹.
6 Although there is no data available for deep sea fish species, considering the relatively
7 short half-life of APs in fish bile of shallow water fish, the detected AP residues in *A.*
8 *rostratus* may also represent signals of ongoing and not historic exposure to these
9 compounds ⁴². Regardless, the detection of APs in bile of fish sampled at up to 2000 m
10 depth, clearly shows that these contaminants are transported to the deep-sea and
11 potentially taken up by deep-sea organisms.

12 Statistical analyses revealed that biliary NP ($t = -2.7$ $p = 0.012$) and OP levels ($t = -5.6$
13 $p < 0.0001$) were significantly higher in samples from WM compared to CS. Although
14 ΣOH-PAH levels also appeared higher at WM compared to CS, no significant
15 differences in OH-PAH levels were detected between the two regions (Table 2). These
16 results, at least for APs, indicate higher contaminant exposure in fish collected at WM
17 compared to those from the CS, potentially reflecting a higher contaminant input to that
18 area. This apparent pollution difference between the two sites is somewhat surprising
19 considering that the WM area is relatively distant from the coastal mainland and is
20 thought to present relatively low contamination levels as no major industrial pollution
21 sources are known to exist in that area ⁴³. The main pollution sources at the Balearic
22 Islands include shipping-, agricultural- and tourism-related activities ⁴⁴, potentially
23 causing the release of APs into the aquatic environment through the use of surfactant-
24 containing pesticides, cleaning products, detergents as well as household and personal
25 care products ¹⁰.

26 However, it is also noteworthy that samples from CS and WM were collected at
27 different times, namely in February and in May 2009, respectively. To what extent this
28 time lag influenced the pollutant exposure levels in *A. rostratus* is difficult to assess.

29 **Gene expression and biomarker responses**

30 The gene expression of EROD, CAT, GR and Cu/Zn-SOD exhibited significantly
31 higher expression levels in male fish at the WM than at the CS site, but not in females

(Figure 2). The transcriptomic response pattern is in accordance with the biliary PAH and AP metabolite profile, indicating higher pollution exposure in fish from the WM compared to those from CS. Previous studies reported an induction of the gene expression of CYP1A⁴⁵⁻⁴⁸, as well as antioxidant enzymes, such as CAT and SOD, in fish exposed to PAHs⁴⁹. Moreover, APs have also been shown to enhance oxidative stress in Atlantic cod⁵⁰, suggesting that the induced antioxidant responses at the gene transcription level could reflect the higher PAH and/or AP exposure in fish from the WM.

However, it should be noted that the induction levels observed in the present study, ranging from 63-181% in males, are relatively low, as compared to, for instance, a 5-fold difference in CYP1A expression between mummichogs (*Fundulus heteroclitus*) from a PAH contaminated and a reference site⁴⁵. Similarly, George et al.⁴⁸ observed 50-fold difference in CYP1A mRNA levels between European flounders (*Platichthys flesus*) collected from an unpolluted and a heavily-contaminated estuary in the UK. Both of these field studies were conducted in heavily polluted estuarine environments. A less marked difference in gene transcript levels between sites in this study is in accordance with the small difference in biliary PAH metabolites, but also the simultaneous exposure to other compounds such as APs, which may downregulate CYP1A expression via crosstalk between the estrogen receptor (ER) and the aryl hydrocarbon receptor (AhR)⁵¹⁻⁵⁴.

With regard to the expression level of Vtg, a 35-fold induction was observed in males from WM compared to those from CS, while females exhibited similar levels at both sites (Figure 2). The fact that Vtg expression in male fish is usually related to exposure to estrogenic contaminants and not affected by their maturity stage^{23, 55} suggests that endocrine-disrupting effects may have occurred in male *A. rostratus* up to depths of 2000 m as a result of higher AP exposure or other endocrine-disrupting chemicals. The lack of Vtg induction in female fish following AP exposure suggests that the presence of estrogenic sex hormones potentially masked the contaminant exposure signal. In some fish species, female Vtg levels increases up to a million-fold during the seasonal reproductive cycle²³, which is also reflected in the 3 to 4 orders of magnitude higher levels in female *A. rostratus* compared to males (Figure 2). The magnitude of the Vtg response detected in male fish is comparable to previous results from field studies

1 conducted in heavily impacted freshwater and coastal environments. For instance, the
2 previously mentioned study by George et al.⁴⁸ found 5-20 times higher Vtg mRNA
3 levels in male flounder from a highly polluted estuary. Similarly, Burki et al.⁵⁶
4 observed no induction of Vtg mRNA in feral, but a 10-fold increase in caged brown
5 trout (*Salmo trutta*) sampled in a Swiss river downstream of a sewage treatment plant.
6 These findings further highlight the importance of the present results, indicating a
7 significant exposure of *A. rostratus* from WM to estrogenic compounds.

8 The fact that male fish showed a more marked transcriptomic stress response than
9 females for the previously discussed biomarker genes (Figure 2) is in accordance with
10 previous studies that reported different effects of estrogenic compounds on male and
11 female fish biomarker responses. In particular, Seo et al.⁵⁷ reported gender differences
12 in the expressions of androgen and estrogen receptor genes in killfish (*Rivulus*
13 *marmoratus*) after NP exposure. Moreover, alkylphenol treatment resulted in different
14 redox status modulations in male and female Atlantic cod (*Gadus morhua*)⁵⁰. Similarly,
15 the laboratory waterborne exposure of fathead minnow (*Pimephales promelas*) to the
16 estrogen 17 β -estradiol resulted in differential response patterns between sexes for a
17 number of genes⁵⁸. Another interesting finding is the fact that expression levels of all
18 five biomarker genes exhibited significant positive relationships in males, while in
19 females the antioxidant genes CAT, Cu/Zn-SOD and GR were correlated among each
20 other and a significant relationship between CYP1A and CAT was observed (Table 3).
21 Moreover, Vtg did not correlate with any other biomarker gene in females, suggesting
22 that, in contrast to males, its expression in females was not related to estrogenic
23 contaminant exposure due to the overriding signal of gonad developmental processes.
24 Overall, this result further highlights the importance of determining potential
25 differences in pollution responses between sexes when conducting biomonitoring
26 studies. In our case, male *A. rostratus* appear to be more adequate for monitoring
27 purposes than females due to their higher responsiveness at the gene expression level
28 and the fact that Vtg mRNA in males can be used as marker for estrogenic exposure.

29 In contrast to gene expression results, biomarker enzyme activities, with the exception
30 of SOD, did not differ significantly between sites for both genders (Figure 3).
31 Furthermore, no significant relationships were detected between gene expression and
32 catalytic activities of a specific biomarker. In general, these biomarker responses vary

1 correspondingly and correlate between each other, with modulations at the gene
2 expression level usually preceding changes in enzymatic activities ⁵⁹. However, some
3 studies have reported a lack of correlation between transcriptomic and catalytic
4 responses, potentially due to differential regulatory mechanisms and the delayed timing
5 of post-transcriptional responses and protein synthesis following pollutant exposure ⁶⁰.
6 Particularly in field conditions, where organisms are typically exposed to complex
7 contaminant mixtures over long time-periods, the dissociation between transcriptional
8 and catalytic responses may even be more pronounced. For instance, several studies
9 have documented differential response trends between transcript expression of genes
10 and the catalytic activities of their respective products, including CYP1A gene
11 expression and EROD activity ^{48, 61-63} as well as antioxidant gene expression and their
12 enzyme activities ^{60, 64}.

13 Furthermore, as mentioned for the biliary metabolite analysis, the time lag between
14 sampling periods may have influenced the biomarker response pattern. In fact, in a
15 previous studies we found significant seasonal variation in enzyme activities of *A.*
16 *rostratus*, including EROD and antioxidant responses ⁶⁵. However, the seasonal pattern
17 appeared to be mainly related to fluctuations in reproductive activity and was mainly
18 observed in females. In the present study, maturity stages were similar between the
19 individuals sampled at both sites, particularly in females no differences in gonad
20 development between CS and WM were observed (see Table 1). Thus, it is likely that
21 the difference in timing of sampling did not affect the biomarker levels in *A. rostratus*.

22 The present study has shown that deep-sea fish dwelling at depths up to 2000 m were
23 exposed to PAHs and APs, with higher metabolite levels detected in bile samples from
24 the WM region. To our knowledge, this is the first study showing significant exposure
25 to AP in a deep-sea fish species. Moreover, Vtg mRNA levels were significantly
26 induced in males from WM, showing that endocrine-disrupting effects, potentially
27 related to AP exposure, may be occurring in such remote environments as the deep-sea.
28 In accordance with chemical results, gene transcript levels also indicated higher
29 pollutant exposure in male fish sampled at WM compared to those from CS area.

30

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Supporting Information

Details on materials and methods for the gene expression analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

1. Scheringer, M.; Salzmann, M.; Stroebe, M.; Wegmann, F.; Fenner, K.; Hungerbühler, K., Long-range transport and global fractionation of POPs: insights from multimedia modeling studies. *Environ. Pollut.* **2004**, *128* (1-2), 177-188.

2. Dachs, J.; Lohmann, R.; Ockenden, W. A.; Méjanelle, L.; Eisenreich, S. J.; Jones, K. C., Oceanic Biogeochemical Controls on Global Dynamics of Persistent Organic Pollutants. *Environ. Sci. Technol.* **2002**, *36* (20), 4229-4237.

3. Ballschmiter, K. H.; Froescheis, O.; Jarman, W. M.; Caillet, G., Contamination of the deep-sea. *Mar. Pollut. Bull.* **1997**, *34* (5), 288-289.

4. Froescheis, O.; Looser, R.; Cailliet, G. M.; Jarman, W. M.; Ballschmiter, K., The deep-sea as a final global sink of semivolatile persistent organic pollutants? Part I: PCBs in surface and deep-sea dwelling fish of the North and South Atlantic and the Monterey Bay Canyon (California). *Chemosphere* **2000**, *40* (6), 651-660.

5. Wania, F.; Daly, G. L., Estimating the contribution of degradation in air and deposition to the deep sea to the global loss of PCBs. *Atmos. Environ.* **2002**, *36* (36-37), 5581-5593.

6. Bouloubassi, I.; Mejanelle, L.; Pete, R.; Fillaux, J.; Lorre, A.; Point, V., PAH transport by sinking particles in the open Mediterranean Sea: A 1 year sediment trap study. *Mar. Pollut. Bull.* **2006**, *52* (5), 560-571.

7. Solé, M.; Porte, C.; Albaiges, J., Hydrocarbons, PCBs and DDT in the NW Mediterranean deep-sea fish *Mora moro*. *Deep-Sea Res. Part I Oceanogr. Res. Pap.* **2001**, *48* (2), 495-513.

8. Escartin, E.; Porte, C., Hydroxylated PAHs in bile of deep-sea fish. Relationship with xenobiotic metabolizing enzymes. *Environ. Sci. Technol.* **1999**, *33* (16), 2710-2714.

9. Ying, G.-G.; Williams, B.; Kookana, R., Environmental fate of alkylphenols and alkylphenol ethoxylates—a review. *Environ. Int.* **2002**, *28* (3), 215-226.

10. David, A.; Fenet, H.; Gomez, E., Alkylphenols in marine environments: Distribution monitoring strategies and detection considerations. *Mar. Pollut. Bull.* **2009**, *58* (7), 953-960.

11. Nimrod, A. C.; Benson, W. H., Environmental Estrogenic Effects of Alkylphenol Ethoxylates. *Crit. Rev. Toxicol.* **1996**, *26* (3), 335-364.

12. Kannan, N.; Yamashita, N.; Petrick, G.; Duinker, J. C., Polychlorinated biphenyls and nonylphenols in the sea of Japan. *Environ. Sci. Technol.* **1998**, *32* (12), 1747-1753.

13. Beyer, J.; Jonsson, G.; Porte, C.; Krahn, M. M.; Ariese, F., Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environ. Toxicol. Pharmacol.* **2010**, *30* (3), 224-244.

14. van der Oost, R.; Beyer, J.; Vermeulen, N. P. E., Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **2003**, *13* (2), 57-149.

15. Whyte, J. J.; Jung, R. E.; Schmitt, C. J.; Tillitt, D. E., Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Crit. Rev. Toxicol.* **2000**, *30* (4), 347-570.

16. Goksøyr, A.; Förlin, L., The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquat. Toxicol.* **1992**, *22* (4), 287-311.

17. Winston, G. W.; Di Giulio, R. T., Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* **1991**, *19* (2), 137-161.

18. Valavanidis, A.; Vlahogianni, T.; Dassenakis, M.; Scoullos, M., Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.* **2006**, *64* (2), 178-189.
19. Matozzo, V.; Gagné, F.; Marin, M. G.; Ricciardi, F.; Blaise, C., Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environ. Int.* **2008**, *34* (4), 531-545.
20. Copeland, P. A.; Sumpter, J. P.; Walker, T. K.; Croft, M., Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri richardson*) at various stages of the reproductive cycle. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **1986**, *83* (2), 487-493.
21. Vetillard, A.; Bailhache, T., Effects of 4-n-Nonylphenol and Tamoxifen on Salmon Gonadotropin-Releasing Hormone, Estrogen Receptor, and Vitellogenin Gene Expression in Juvenile Rainbow Trout. *Toxicol. Sci.* **2006**, *92* (2), 537-544.
22. Arukwe, A.; Røe, K., Molecular and cellular detection of expression of vitellogenin and zona radiata protein in liver and skin of juvenile salmon (*Salmo salar*) exposed to nonylphenol. *Cell Tissue Res.* **2008**, *331* (3), 701-712.
23. Sumpter, J. P.; Jobling, S., Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* **1995**, *103* (SUPPL. 7), 173-178.
24. Min, J.; Lee, S.-K.; Gu, M. B., Effects of endocrine disrupting chemicals on distinct expression patterns of estrogen receptor, cytochrome P450 aromatase and p53 genes in oryzias latipes liver. *J. Biochem. Mol. Toxicol.* **2003**, *17* (5), 272-277.
25. Arukwe, A.; Kullman, S. W.; Hinton, D. E., Differential biomarker gene and protein expressions in nonylphenol and estradiol-17 β treated juvenile rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2001**, *129* (1), 1-10.
26. Sardà, F.; Cartes, J. E.; Company, J. B.; Albiol, A., A Modified Commercial Trawl Used to Sample Deep-Sea Megabenthos. *Fish. Sci.* **1998**, *64* (3), 492-493.
27. Brown-Peterson, N. J.; Wyanski, D. M.; Saborido-Rey, F.; Macewicz, B. J.; Lowerre-Barbieri, S. K., A Standardized Terminology for Describing Reproductive Development in Fishes. *Mar. Coast. Fish.* **2011**, *3* (1), 52-70.
28. Cartes, J. E.; Abello, P.; Lloris, D.; Carbonell, A.; Torres, P.; Maynou, F.; de Sola, L. G., Feeding guilds of western Mediterranean demersal fish and crustaceans: an analysis based in a spring survey. *Sci. Mar.* **2002**, *66* (Suppl. 2), 209-220.
29. Morales-Nin, B.; Massutí, E.; Stefanescu, C., Distribution and biology of *Alepocephalus rostratus* from the Mediterranean Sea. *J. Fish Biol.* **1996**, *48* (6), 1097-1112.
30. Fernandes, D.; Andreu-Sánchez, O.; Bebianno, M. J.; Porte, C., Assessment of pollution along the Northern Iberian shelf by the combined use of chemical and biochemical markers in two representative fish species. *Environ. Pollut.* **2008**, *155* (2), 327-335.
31. Koenig, S.; Fernandez, P.; Company, J. B.; Huertas, D.; Solé, M., Are deep-sea organisms dwelling within a submarine canyon more at risk from anthropogenic contamination than those from the adjacent open slope? A case study of Blanes canyon (NW Mediterranean). *Prog. Oceanogr.* **2012**, *Special Issue: Mediterranean Deep Canyons*, In Press.
32. Aebi, H., Catalase. In *Methods of enzymatic analysis*, Bergmeyer, H. U., Ed. Academic Press: London, 1974 pp 671-684.
33. Carlberg, I.; Mannervik, B., Glutathione reductase. *Method. Enzymol.* **1985**, *113*, 484-490.

34. Kopecka-Pilarczyk, J.; Correia, A. D., Biochemical response in gilthead seabream (*Sparus aurata*) to in vivo exposure to pyrene and fluorene. *J. Exp. Mar. Biol. Ecol.* **2009**, *372* (1-2), 49-57.

35. Burke, M. D.; Mayer, R. T., Ethoxyresorufin: direct fluorimetric assay of a microsomal o-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **1974**, *2* (6), 583-588.

36. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72* (1-2), 248-254.

37. Livak, K. J.; Schmittgen, T. D., Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* **2001**, *25* (4), 402-408.

38. Arukwe, A., Toxicological Housekeeping Genes: Do They Really Keep the House? *Environ. Sci. Technol.* **2006**, *40* (24), 7944-7949.

39. Filby, A.; Tyler, C., Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. *BMC Mol. Biol.* **2007**, *8* (1), 10.

40. Ruddock, P. J.; Bird, D. J.; McCalley, D. V., Bile Metabolites of Polycyclic Aromatic Hydrocarbons in Three Species of Fish from the Severn Estuary. *Ecotoxicol. Environ. Saf.* **2002**, *51* (2), 97-105.

41. Martin-Skilton, R.; Lavado, R.; Thibaut, R.; Minier, C.; Porte, C., Evidence of endocrine alteration in the red mullet, *Mullus barbatus* from the NW Mediterranean. *Environ. Pollut.* **2006**, *141* (1), 60-68.

42. Beyer, J.; Sundt, R. C.; Sanni, S.; Sydnes, M. O.; Jonsson, G., Alkylphenol metabolites in fish bile as biomarkers of exposure to offshore oil industry produced water in feral fish. *J. Toxicol. Environ. Health Part A* **2011**, *74* (7-9), 569-581.

43. Baumard, P.; Budzinski, H.; Michon, Q.; Garrigues, P.; Burgeot, T.; Bellocq, J., Origin and Bioavailability of PAHs in the Mediterranean Sea from Mussel and Sediment Records. *Estuarine, Coastal and Shelf Science* **1998**, *47* (1), 77-90.

44. Box, A.; Sureda, A.; Galgani, F.; Pons, A.; Deudero, S., Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2007**, *146* (4), 531-539.

45. Roling, J. A.; Bain, L. J.; Baldwin, W. S., Differential gene expression in mummichogs (*Fundulus heteroclitus*) following treatment with pyrene: comparison to a creosote contaminated site. *Mar. Environ. Res.* **2004**, *57* (5), 377-395.

46. Benedetti, M.; Martuccio, G.; Fattorini, D.; Canapa, A.; Barucca, M.; Nigro, M.; Regoli, F., Oxidative and modulatory effects of trace metals on metabolism of polycyclic aromatic hydrocarbons in the Antarctic fish *Trematomus bernacchii*. *Aquat. Toxicol.* **2007**, *85* (3), 167-175.

47. Bilbao, E.; Raingeard, D.; de Cerio, O. D.; Ortiz-Zarragoitia, M.; Ruiz, P.; Izagirre, U.; Orbea, A.; Marigómez, I.; Cajarville, M. P.; Cancio, I., Effects of exposure to Prestige-like heavy fuel oil and to perfluorooctane sulfonate on conventional biomarkers and target gene transcription in the thicklip grey mullet *Chelon labrosus*. *Aquat. Toxicol.* **2010**, *98* (3), 282-296.

48. George, S.; Gubbins, M.; MacIntosh, A.; Reynolds, W.; Sabine, V.; Scott, A.; Thain, J., A comparison of pollutant biomarker responses with transcriptional responses in European flounders (*Platichthys flesus*) subjected to estuarine pollution. *Mar. Environ. Res.* **2004**, *58* (2-5), 571-575.

49. Nahrgang, J.; Camus, L.; Gonzalez, P.; Goksøyr, A.; Christiansen, J. S.; Hop, H., PAH biomarker responses in polar cod (*Boreogadus saida*) exposed to

- benzo(a)pyrene. *Aquat. Toxicol.* **2009**, *94* (4), 309-319.
50. Hasselberg, L.; Meier, S.; Svandal, A., Effects of alkylphenols on redox status in first spawning Atlantic cod (*Gadus morhua*). *Aquat. Toxicol.* **2004**, *69* (1), 95-105.
51. Arukwe, A.; Förlin, L.; Goksøyr, A., Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. *Environ. Toxicol. Chem.* **1997**, *16* (12), 2576-2583.
52. Sturve, J.; Hasselberg, L.; Fälth, H.; Celander, M.; Förlin, L., Effects of North Sea oil and alkylphenols on biomarker responses in juvenile Atlantic cod (*Gadus morhua*). *Aquat. Toxicol.* **2006**, *78*, Supplement (0), S73-S78.
53. Navas, J. M.; Segner, H., Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. *Chem.-Biol. Interact.* **2001**, *138* (3), 285-298.
54. Celander, M. C., Cocktail effects on biomarker responses in fish. *Aquat. Toxicol.* **2011**, *105* (3-4, Supplement), 72-77.
55. Hutchinson, T. H.; Ankley, G. T.; Segner, H.; Tyler, C. R., Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. *Environ. Health Perspect.* **2006**, *114 Suppl 1*, 106-114.
56. Burki, R.; Vermeirssen, E. L. M.; Körner, O.; Joris, C.; Burkhardt-Holm, P.; Segner, H., Assessment of estrogenic exposure in brown trout (*Salmo trutta*) in a Swiss midland river: Integrated analysis of passive samplers, wild and caged fish, and vitellogenin mRNA and protein. *Environ. Toxicol. Chem.* **2006**, *25* (8), 2077-2086.
57. Seo, J. S.; Lee, Y.-M.; Jung, S.-O.; Kim, I.-C.; Yoon, Y.-D.; Lee, J.-S., Nonylphenol modulates expression of androgen receptor and estrogen receptor genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*. *Biochem. Biophys. Res. Commun.* **2006**, *346* (1), 213-223.
58. Filby, A. L.; Thorpe, K. L.; Tyler, C. R., Multiple molecular effect pathways of an environmental oestrogen in fish. *J. Mol. Endocrinol.* **2006**, *37* (1), 121-134.
59. Piña, B.; Casado, M.; Quirós, L., Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. *Trends Anal. Chem.* **2007**, *26* (11), 1145-1154.
60. Regoli, F.; Giuliani, M. E.; Benedetti, M.; Arukwe, A., Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquat. Toxicol.* **2011**, *105* (3-4, Supplement), 56-66.
61. Tom, M.; Shmul, M.; Shefer, E.; Chen, N.; Slor, H.; Rinkevich, B.; Herut, B., Quantitative evaluation of hepatic cytochrome P4501A transcript, protein, and catalytic activity in the striped sea bream (*Lithognathus mormyrus*). *Environ. Toxicol. Chem.* **2003**, *22* (9), 2088-2092.
62. Trisciani, A.; Corsi, I.; Torre, C. D.; Perra, G.; Focardi, S., Hepatic biotransformation genes and enzymes and PAH metabolites in bile of common sole (*Solea solea*, Linnaeus, 1758) from an oil-contaminated site in the Mediterranean Sea: A field study. *Mar. Pollut. Bull.* **2011**, *62* (4), 806-814.
63. Kammann, U.; Lang, T.; Berkau, A.-J.; Klempt, M., Biological effect monitoring in dab (*Limanda limanda*) using gene transcript of CYP1A1 or EROD—a comparison. *Environ. Sci. Pollut. Res.* **2008**, *15* (7), 600-605.
64. Nahrgang, J.; Camus, L.; Gonzalez, P.; Jönsson, M.; Christiansen, J. S.; Hop, H., Biomarker responses in polar cod (*Boreogadus saida*) exposed to dietary crude oil. *Aquat. Toxicol.* **2010**, *96* (1), 77-83.
65. Koenig, S.; Solé, M., Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms. *Mar. Environ. Res.* **2012**, *79* (0), 122-131.

Table 1 Biological characteristics and sample sizes (N) of *Alepocephalus rostratus* sampled at two sites within the Mediterranean sea. Maturity scale are given as range based on five-point scale by Brown-Peterson et al., (2011) ²⁷

Site	Sex	Depth (m)	Maturity	N	Size (mm)
Catalan slope (CS)	F	1500	2-4	9	326 ± 15
	M	1500	2-4	10	302 ± 7
Western Mediterranean (WM)	F	1200	2-4	5	320 ± 24
	M	2000	1-3	6	223 ± 17

Table 2 Biliary metabolites detected in *Alepocephalus rostratus* at two sampling sites within the Western Mediterranean basin: Catalan slope (CS) and western Mediterranean (WM). Values represent mean (ng/g bile) ± S.E.M. (min.-max.). Asterisk denotes significant difference between sites based on Student's t-test (p < 0.05).

Bile metabolite	CS (n = 16)	WM (n = 9)
1-naphthol	74.4 ± 8.2 (15.5 - 122.3)	107.7 ± 26.6 (33.9 - 287.3)
2-naphthol	1.7 ± 0.5 (n.d. - 3.5)	3.2 ± 0.6 (n.d. - 6.0)
9-fluorenol	4.1 ± 0.7 (n.d. - 7.7)	5.5 ± 0.6 (3.7 - 8.3)
9-phenanthrol	n.d.	n.d.
1-pyrenol	0.6 ± 0.3 (n.d. - 4.6)	n.d.
ΣOH-PAHs	80.9 ± 8.2 (21.1 - 122.3)	116.4 ± 27.4 (40.1 - 300.3)
4-nonylphenol*	42.5 ± 5.8 (17.4 - 76.0)	67.1 ± 6.7 (42.3 - 102.7)
4-tert-octylphenol*	7.9 ± 0.7 (4.9 - 15.1)	14.2 ± 0.8 (9.5 - 17.3)

Table 3 Spearman rank correlations between mRNA levels of 5 different biomarker genes in male (underlined) and female *A. rostratus*

Gene	CYP1A	CAT	Cu/Zn-SOD	GR	Vtg
CYP1A		$\rho = 0.78^{***}$	$\rho = 0.71^{**}$	$\rho = 0.55^*$	$\rho = 0.63^{***}$
CAT	$\rho = 0.78^{**}$		$\rho = 0.76^{***}$	$\rho = 0.61^*$	$\rho = 0.69^{**}$
Cu/Zn-SOD	n.s.	$\rho = 0.86^{***}$		$\rho = 0.81^{***}$	$\rho = 0.78^{***}$
GR	n.s.	$\rho = 0.65^*$	$\rho = 0.63^*$		$\rho = 0.56^*$
Vtg	n.s.	n.s.	n.s.	n.s.	

* p<0.05; **p<0.01, ***p<0.001
n.s. = not significant

Figure captions

Fig. 1 Map of sampling sites within the Mediterranean Sea. The map was created using the Ocean Data View (ODV) software package by Schlitzer, R., Ocean Data View, <http://odv.awi.de>, 2010.

Fig.2 Gene expression for female and male *A. rostratus* from the Catalan slope (CS) (M: n=10; F: n=9) and the Western Mediterranean (WM) (M: n=6; F: n=5) areas. Values shown are mean $\pm$ S.E.M. Asterisk denotes significant difference between sites ($p < 0.05$).

Fig.3 Biomarker enzyme activities for female and male *A. rostratus* from the Catalan slope (CS) (M: n=10; F: n=9) and the Western Mediterranean (WM) (M: n=6; F: n=5) areas. Values shown are mean $\pm$ S.E.M. Asterisk denotes significant difference between sites ($p < 0.05$).

Fig.1

Fig.2

Fig.3

TOC/Abstract art