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Origin And Distribution Of Polycyclic Aromatic Hydrocarbon Pollution In Sediment And Fish In The Biosphere'S Reserve Of Urdaibai (Bay Of Biscay, Basque Country, Spain)

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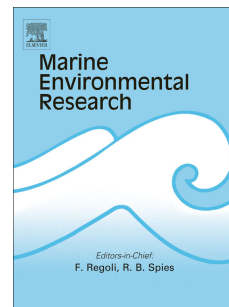
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**ORIGIN AND DISTRIBUTION OF POLYCYCLIC AROMATIC
HYDROCARBON POLLUTION IN SEDIMENT AND FISH IN THE
BIOSPHERE'S RESERVE OF URDAIBAI (BAY OF BISCAY, BASQUE
COUNTRY, SPAIN)**

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Abstract

1 The Urdaibai estuary is an UNESCO's biosphere reserve impacted by recreational,
2 agricultural and industrial activities. Polycyclic aromatic hydrocarbons (PAHs) are
3 major pollutants in Urdaibai, and their control and the identification of their sources is
4 central on the preservation of the area. Chemical analysis by GC-MS showed a
5 significant pollution by PAHs in Urdaibai sediment samples, mainly from pyrolytic
6 sources, with minor contributions from oil spills. Measurement of the dioxin-like
7 activity using a yeast-based bioassay showed an excess of biological activity in
8 sediment samples from the inner part of the estuary. Analysis of PAH metabolites in
9 bile of thicklip grey mullet (*Chelon labrosus*) showed a large excess of 2-naphthol over
10 1-naphthol or 1-pyrenol, suggesting a specific contamination in the inner Urdaibai
11 estuary by some industrial process. Therefore, the combination of these three techniques
12 defined different PAH pollution sources in Urdaibai: A major pyrolytic origin,
13 occasional oil spills, and specific industrial activities.
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27 *Keywords:* Dioxin-like activity, Recombinant yeast assay, GC/MS, PAH metabolites
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1. Introduction

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4 The estuary of Urdaibai (43°23'N – 2°41'W) is located in the Bay of Biscay, in the
5 Northern Iberian Peninsula (Figure 1). It is 13 km long and an average width of 500m,
6 and presents a limited maritime traffic. The main human activity in the zone is
7 agriculture but a variety of industries can be found such as metallurgic industries,
8 shipyards, treatment of surfaces, dyes and manufacture of cutlery, concentrated in the
9 surroundings of the main town in the area, Gernika-Lumo. Due to its unique landscape
10 and to its good balance between human activities and the natural environment, it was
11 declared a Biosphere's Reserve by UNESCO in 1984.
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18 There are several environmental menaces looming over the Urdaibai ecosystem.
19 Contamination by polycyclic aromatic hydrocarbons (PAHs) is of special concern, as
20 they can be derived from both human activities (industrial byproducts, incomplete
21 combustion of carburants) or from oil spills, either internal (shipping, carburant
22 handling) or external. In this regard, massive oil spills, like the Prestige spill in 2002,
23 are known to affect the estuary in the recent past (Cortazar et al., 2008; Orbea et al.,
24 2006). Therefore, the continuous surveillance of PAHs pollution and the identification
25 of its possible sources is central on the maintaining of the environmental health of the
26 Urdaibai estuary. The present work addresses these issues using a combination of
27 chemical analyses and bioassays directed to determine PAHs levels and composition in
28 sediments, evaluate their biological activity, and analyze metabolites diagnostic for their
29 origin and possible sources.
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40 Due to their hydrophobicity, PAHs accumulate both in sediments and in the fat
41 of exposed animals. They are considered hazardous for human health and a number of
42 them are known carcinogenic compounds (Freire et al., 2009; IARC, 1983). PAHs
43 constitute a very extended family of compounds, from which regulatory bodies have
44 defined more or less comprehensive lists of priority compounds. The sixteen
45 compounds listed by the US Environmental Protection Agency (EPA) or the eight ones
46 included by the Water Framework Directive of the European Union (EU WFD) are used
47 to define annual average and maximum allowable concentrations in surface waters (EC,
48 2008). In contrast, no comparable quality standards for sediments have been defined
49 yet, despite of the tendency of these compounds to accumulate in hydrophobic
50 compartments. In this work, we analyze the sixteen EPA PAHs along the Urdaibai
51 estuary to identify areas of accumulation and to infer possible sources.
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1 The carcinogenic effect of different PAHs is a consequence of the ectopic up
2 regulation of the oxidative metabolism in liver and other tissues from vertebrates. This
3 effect is mediated by the binding of the pollutant to the aryl hydrocarbon receptor (AhR)
4 (Gonzalez and Fernández-Salguero, 1998; Shimizu et al., 2000; van der Oost et al.,
5 2003). The capacity of substances or environmental samples to activate the AhR system
6 (the so-called dioxin-like activity, as dioxins are known to bind to AhR with extremely
7 high affinity) can be quantified by a variety of aryl hydrocarbon-responsive reporter
8 assays based on either in vivo animal tests (Willey et al., 1998) or different vertebrate
9 cell lines (Villeneuve et al., 2002). Yeast-based bioassays represent a fast and cheaper
10 alternative, although reports of their use for detection of AhR ligands in environmental
11 samples are still scarce (Boronat et al., 2009; Lee et al., 2005; Murahashi et al., 2007).
12 Yeasts lack AhR or any equivalent receptor, but their genetic make-up can be easily
13 modified to reconstruct at least partially the mammalian pathway (Miller, 1999; Miller,
14 1997). In the present study we used a yeast strain expressing the human AhR and the
15 cofactor ARNT to activate a reporter gene (β -galactosidase) upon the presence of AhR
16 ligands in the cell culture (Miller, 1997). In this genetic setup, the production of the
17 reporter gene is diagnostic for the presence of AhR ligands in the medium, and can be
18 quantified as an enzymatic activity with high precision (Noguerol et al., 2006a). With
19 these tools, we determined AhR ligand activity in sediment samples from Urdaibai and
20 compared these values with the observed chemical burden, to evaluate the importance
21 of PAH pollution in the total toxicity of the sediments.

22 Fish living in PAH-contaminated environments absorb PAHs through the body
23 surface and gills or by ingestion of contaminated sediment or food (Orbea et al., 2006;
24 Raingard et al., 2006; Varanasi et al., 1989). In this work, we assessed the exposure to
25 PAHs in the thicklip grey mullet (*Chelon labrosus*, Mugilidae), a coastal fish present in
26 eastern Atlantic Ocean and abundant in Urdaibai and other estuaries. This species is a
27 good sentinel of human impact, as it feeds mainly on benthic diatoms, algae, small
28 invertebrates and detritus. Direct measure of PAHs in tissues is impaired by the high
29 capacity to metabolize and excrete PAHs of fish liver (Meador et al., 1995).
30 Quantitation of PAHs excreted through the bile as conjugated metabolites is a suitable
31 alternative to assess PAHs exposure in fish, both in the field and in laboratory
32 experiments, since bile metabolites can easily be detected with high precision and
33 sensitivity (Escartin and Porte, 1999; Fernandes et al., 2008a; Fernandes et al., 2002;
34 Fernandes et al., 2008b; Yu et al., 1995). This methodology not only allows an

1 estimation of the total PAH burden, but it also reveals information of the composition of
2 this pollution, a central issue in the evaluation of the possible pollution sources.
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5 **2. Materials and Methods**

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7 *2.1 Study area and sample collection*

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12 Surface sediment samples (one per site) were collected in April 2007, October 2007,
13 and April 2008, in order to explore temporal trends, at six localities within the Urdaibai
14 estuary, from its upstream limit to the mouth (Figure 1): Gernika, Murueta, Arteaga,
15 Txatxarramendi, Mundaka and Laida. Murueta is a small town in the middle part of the
16 estuary, where a medium size shipyard is placed. Arteaga is a small rural village,
17 whereas Txatxarramendi is a small island in the outer part of the estuary. Mundaka is a
18 small town in the mouth of the Oka River whose main activities are fisheries,
19 agriculture and tourism. Laida is a beach at the mouth of the estuary, in front of
20 Mundaka. For comparison purposes, sediments were also sampled in the leisure harbour
21 of Arriluze, in the Abra estuary in the Basque Coast (43°23'N - 3°W, Figure 1), a site
22 known by its high levels of pollution by PAHs (Cearreta et al., 2004). Surface sediment
23 samples, collected in precleaned amber-glass bottles, were transported to the laboratory
24 in refrigerated containers, and then frozen at -20 °C.
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36 Fish (thicklip grey mullet *Chelon labrosus*) were sampled at approximately 1
37 Km distance from Gernika-Lumo urban center, near a sewage treatment plant, and in
38 the Arriluze harbor, to compare PAH burden inside and outside Urdaibai. Six animals
39 were captured in each site and campaign (36 animals in total). Fish were anaesthetized
40 by immersion in a saturated solution of aminobenzocaine. Length (29.5±5.5 cm) and
41 weight (395.9±184.6 g) of each fish were recorded, and the bile was taken, frozen in
42 liquid nitrogen and stored at -80°C until analysis.
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51 *2.2 Analysis of PAHs in sediments*

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54 Sediments samples were lyophilized during 48 h (10⁻² mbar vacuum) (Lioalfa, Telstar,
55 Spain). The freeze-dried material was ground and homogenized using a mortar. Samples
56 were sieved through 500 and 120 µm mesh to obtain an homogeneous sediment material
57 and stored in precleaned glass bottles at -20 °C until extraction. Analysis of PAHs in the
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sediments was performed as described (Martinez et al., 2004). Sediment samples (1 g) were extracted by ultrasonication with 3×20 mL of a mixture of hexane:dichloromethane (1:1), and the resulting extracts were cleaned-up by solid phase extraction (SPE) using neutral alumina, reconstituted in 250 µL hexane, and analyzed of the resulting purified extracts by gas chromatography/mass spectrometry (GC/MS) with electron impact ionisation (70 eV) acquiring 3 characteristic ions per compound. Quantitation, based on peak areas, was performed by the internal standard method using surrogate deuterated standards. This method allows the determination of the 16 EPA PAHs at ng/g levels (limits of detection between 0.1 and 2 ng/g) with satisfactory precision (relative standard deviation calculated from 3 replicates below 20%) and accuracy (recovery percentage above 50% for all compounds except Naphthalene (20%) and Benzo[g,h,i]Perylene (47%). Analysis (n=3) of a certified freshwater harbour sediment material (BCR535) within the sample sequence yielded concentrations matching the certified values with a percentage of error below 12%.

Ratios of the PAH isomers Phenanthrene to Anthracene (Ph/An), Fluoranthene to Pyrene (Fl/Py) and Benzo[a]Anthracene to Chrysene (BaA/Chrys) were calculated as indicators of PAH origin. Values of Ph/An>50, Fl/Pyr<1 and BaA/Chrys<1 are typical of samples of petrogenic (oil spill) origin, whereas the corresponding ratios of samples originated in combustion processes (pyrolytic) are typically Ph/An<10, Fl/Pyr>1 and BaA/Chrys>1 (Karacik et al., 2009; Poster et al., 2000; Webster et al., 2001).

2.3 Recombinant yeast assay

Aryl hydrocarbon receptor (AhR)-based recombinant yeast assays (AhR-RYA) were performed as previously described (Noguerol et al., 2006a; Noguerol et al., 2006b). The YCM1 yeast strain (Miller, 1997) was grown overnight in minimal medium (6.7 g/L) yeast nitrogen base without amino acids plus ammonium sulfate (DIFCO, Basel, Switzerland) supplemented with 0.1 g/L of prototrophic markers as required and with galactose as a carbon source to express AhR and the cofactor ARNT (Miller, 1999). The final culture was adjusted to an optical density (OD) of 0.1 and split into 100 µL aliquots in 96-well polypropylene microtiter plates (NUNC™, Roskilde, Denmark; (Garcia-Reyero et al., 2001) previously silylated by overnight exposure to an atmosphere of dimethylsilane. We performed a serial dilution scheme based on 1:3 dilution steps and using glass microcapillary tubes rather than pipette tips for all liquid

1 transfers, to eliminate contact with plastic surfaces (Garcia-Reyero et al., 2001). Plates
2 were incubated for 4 hours at 30°C under mild shaking. After incubation, 100 µL
3 YPER™ (PIERCE™, Rockford, IL, USA) were added to each well and further
4 incubated at 30°C for 30 min. Afterwards, 100 µL of assay buffer supplemented with
5 0.1% 2- mercaptoethanol and 0.5% of the 4-methylumbelliferone β-D-
6 galactopyranoside (MuGal) solution (both from the FluorAce™ beta-galactosidase
7 Reporter Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA) were added to the lysed
8 cells. After brief centrifugation, plates were read in a Victor3 Wallac spectrofluorometer
9 (Perkin Elmer Inc., Wellesley, MA, USA), at 355nm excitation and 460nm emission
10 wavelengths. Fluorescence was recorded for 15 to 20 min (one measurement per min); β
11 galactosidase activity values were calculated as rates of the increment of arbitrary
12 fluorescence units with time, using standard linear regression methods. To test possible
13 inhibitory activity (false negatives), yeast cultures were incubated for 4h with 2 µM of
14 β-naphthoflavone, added to a 1:30 dilution of each sample, and processed as before. No
15 sample exhibited inhibitory activity high enough to significantly distort the results (not
16 shown). In this particular system, the ratio of false positives is negligible (Miller, 1997;
17 Noguerolet al., 2006a); several negative controls (wells with only vehicle plus yeast
18 culture) were included in each plate. Samples were tested in triplicate. AhR-ligand
19 activity values were calculated as naphthoflavone equivalents, NFEQ, defined as the
20 concentration of β-naphthoflavone required to elicit the same response as the sample in
21 the assay. These values were calculated from dose-response curves by adjusting β
22 galactosidase values to a first-order Hill equation, as described (Noguerolet al., 2006a),
23 using at least 9 determinations for each value.
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44 *2.4 Analysis of PAH metabolites in fish bile*

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47 Hydroxylated PAH metabolites were analyzed by a modification of a previously
48 described method (Escartin and Porte, 1999). Briefly, 100 mg of bile (6 samples per
49 area and sampling campaign) were incubated for 1 h at 40°C in the presence of 1 ml 0.4
50 M acetic acid/sodium acetate buffer pH 5.0, containing 2000 U of β-glucuronidase and
51 50 U of sulfatase. Hydrolyzed metabolites were extracted with 1 ml of ethyl acetate
52 (x3), the extracts recombined and concentrated under a nitrogen stream. Dry residues of
53 bile metabolites were then derivatized by the addition of 100 µl of bis-(trimethylsilyl)
54 trifluoroacetamide (BSTFA), heated for 1 h at 70°C, and further reduced under a
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1 nitrogen stream. Analyses were carried by gas chromatography- mass spectrometry
2 electron impact mode (GC-MS-EI) in a Fisons GC 8000 Series chromatograph
3 interfaced to a Fisons MD800 mass spectrometer using a 30 m x 0.25 mm internal
4 diameter HP-5MS column crosslinked with 5% PH ME siloxane (Hewlett-Packard,
5 USA). The instrument was programmed from 90°C to 140°C at 10°C min⁻¹ and from
6 140°C to 300°C at 4°C min⁻¹, using helium at 80 kPa as carrier gas. The injector
7 temperature was 250°C and the ion source and the analyzer were maintained at 200°C
8 and 250°C, respectively. Just prior to sample analysis, calibration curves were
9 performed with reference compounds and operating in selected ion monitoring mode
10 (SIM). Hydroxylated-PAHs were identified by comparison of retention times and
11 spectra of reference compounds. The silylation derivative ions used for monitoring and
12 quantification of hydroxylated-PAHs were: *m/z* 216 and 201 for 1- and 2-naphthol; *m/z*
13 254 and 165 for 9-fluoreno; *m/z* 266 and 251 for 9-phenanthrol; and *m/z* 290 for 1-
14 pyrenol. Recovery of extraction procedure was higher than 90% for all the compounds
15 tested (1-naphthol, 9-fluoreno and 9-phenanthrol), except for 1-pyrenol which was 85%
16 (Escartin and Porte, 1999). Concentrations are expressed as nanograms per gram of bile.
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30 2.5 Statistics

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34 All statistical calculations, including linear and non-linear regression methods, were
35 performed using the SPSS v. 17 package (SPSS Inc., Chicago, Ill.) Unless otherwise
36 noted, significance levels were set at $p < 0.05$. Normality of distributions was tested with
37 the Kolmogorov-Smirnov Z test. Most variables analyzed showed a non-normal
38 distribution by the Z-test; therefore, non-parametric test were preferred. In cases in
39 which parametric tests were required (e.g., ANOVA plus Tukey's test), they were
40 performed using logarithmic transformants of the data, which followed a normal
41 distribution with uniform variances for all categories tested (Z- and Levene's tests).
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51 3. Results and Discussion

52 3.1.- PAH levels in sediments

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58 Total PAH levels in Urdaibai sediments varied some 50-fold among sampling sites
59 (Table 1, profiles in Figure 2A, bars). Except for Mundaka, which showed an extremely
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1 high variability, results from the three campaigns were similar for all sites, as revealed
2 by K-means cluster analysis (Fig. 2B). The three sites at the inner part of the Urdaibai
3 estuary showed similar, relatively low PAHs burdens, compared to Txatxarramendi and
4 Arriluze sites (Figure 2). The two outer sites, Laia and Mundaka showed low PAH
5 burdens comparable to those from the Urdaibai inner sites, except for the October 2007
6 sample from Mundaka, which we consider directly contaminated with oil (see next
7 paragraph). A similar survey performed in years 2002-2004 recorded PAHs levels
8 between 1,000 and 10,000 ng/g, with some occasional samples largely exceeding these
9 values (Cortazar et al., 2008). These results suggest that PAH levels remained
10 essentially identical since at least 2002, suggesting a continuous, chronic contamination
11 by PAHs, not necessarily linked to episodic oil spills.
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20 Analysis of ratios between PAH isomers indicates a mainly pyrogenic origin of
21 PAHs found in Urdaibai sediments (Table 1). 20 out of 21 samples showed Ph/An ratios
22 well below 10, indicative of a combustion process. Similarly, 14 out of 18 samples
23 showed BaA/Chr ratios over 1, also diagnostic of pyrogenesis (Karacik et al., 2009;
24 Poster et al., 2000; Webster et al., 2001). In contrast, Fl/Pyr ratios below 1 shown by 11
25 out of the 21 samples suggest some contribution from oil spills (petrogenic) in at least
26 some of them (Table 1). Petrogenic pollution, albeit never predominant, seemed to be
27 more important in Gernika, Murueta and in the leisure harbor of Arriluze (Table 1),
28 likely from seafaring and occasional oil spills from different sources. The October 2007
29 sample from Mundaka, which showed the highest PAH contents in all the series, is the
30 only one with a Ph/An ratio close to 50, indicative of petrogenic origin (Table 1, Figures
31 2A and 2B). We concluded that this particular sample may have been contaminated by
32 an oil spill, probably a local one. With the exception of this particular sample, our
33 results indicate that most PAHs found in Urdaibai and Arriluze have a pyrolytic origin,
34 with a possible additional contribution of petrogenic hydrocarbons. Similar conclusion
35 was reached in previous reports of PAH contents in Urdaibai sediments (Cortazar et al.,
36 2008) and in the Istanbul Strait (Karacik et al., 2009), both showing PAHs levels and
37 isomeric ratios similar to the ones observed in our study.
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54 3.2. AhR ligand activity in sediments

55 All samples from Urdaibai sediments were positive for AhR ligand activity, indicating a
56 general contamination by dioxin-like compounds (Figure 2). Maximal activities
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1 corresponded to Txatxarramendi and Arriluze sites, whereas minimal values were found
2 in Mundaka and Laida sites (Figure 2, continuous line). Differences between samples
3 collected at a given site during the three campaigns were relatively small (less than a
4 factor of two), except for some few exceptions (Figure 2). K-means clustering of data
5 clearly distinguished the two outer sites (Mundaka and Laia) as the ones with the lowest
6 AhR-ligand activity, followed by the three Urdaibai inner sites (Gernika, Arteaga and
7 Murueta). In the same analysis, Txatxarramendi appeared as the most contaminated site,
8 surpassing even the Arriluze site (Figure 2B). The Abra estuary is much more heavily
9 industrialised than the Urdaibai estuary, receiving considerable amounts of industrial,
10 urban and agricultural untreated wastewater during about 150 years. Since 1980 a
11 sewage treatment plant was set up and the environmental quality of the estuary started a
12 recovery process (Gorostiaga et al., 2004), although levels of pollution by PAHs and
13 other chemicals are still considered very high (Cearreta et al., 2004). Therefore, the
14 presence in Urdaibai of a site with pollution levels similar to (if not even higher than)
15 those from the Arriluze site, both in terms of chemical content and biological activity,
16 argues for the requirement of a more strict control of pollution in the generally regarded
17 as preserved Urdaibai estuary.

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31 Comparison between AhR ligand activity and PAH burden profiles showed
32 some significant similarities as well as differences. For example, both profiles show an
33 increasing tendency from the innermost Urdaibai site, Gernika, to Txatxarramendi,
34 close to the mouth of the estuary (Figures 2A and 2B, map in Figure 1). This last site
35 showed values for both parameters comparable to the ones from the polluted Arriluze
36 site in both profiles. In contrast, Mundaka and Laida showed a rather poor correlation
37 between chemical and bioassay data, especially for the Mundaka site. In both cases, the
38 observed low AhR ligand activity was at variance with their relatively high PAH
39 burden. These discrepancies were diminished when the chemical data was converted to
40 predicted dioxin-like activity, measured as TCDD equivalents (TEQ, Figure 2,
41 discontinuous line), using relative effector potency (REP) values previously defined for
42 AhR-RYA (Murahashi et al., 2007). By comparing the three profiles in Figure 2, we
43 concluded that sediment samples from Mundaka and Laida were particularly poor in
44 biologically active PAHs. This was particularly significant for the very highly polluted
45 October 2007 Mundaka sample, which shows a very low biological activity.
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1 contain some unidentified AhR ligand to explain their higher-than-predicted dioxin-like
2 activity in the AhR-RYA assay (Figure 2).

3 AhR ligand activity values showed strong positive correlation with the
4 concentration of two groups of individual PAHs: The three-ringed Phenanthrene and
5 Anthracene as one group and the four-ringed Benzo[a]anthracene,
6 Benzo[b]fluoranthene, Benzo[k]fluoranthene and Benzo[a] pyrene as the second one
7 (Table 2). Most of these compounds are known to bind the human AhR and to activate
8 AhR-regulated genes, both *in vitro* and *in vivo* (Murahashi et al., 2007; Villeneuve et
9 al., 2002; Willey et al., 1998). Consistently, we found a good correlation between AhR-
10 RYA results and the predicted TEQ values (Table 2). These results therefore indicate
11 that a substantial fraction of the total AhR ligand activity present in Urdaibai sediments
12 can be explained by their contents in the 16 EPA PAHs.
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23 3.3 PAH metabolites in fish bile

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27 Levels of PAH hydroxylated metabolites in bile of fish captured in Urdaibai and in
28 Arriluze reflected the relative concentration of PAHs found in the sediments from both
29 sites (Figure 3A). Fish collected in Arriluze showed values of PAHs metabolites in bile
30 (734 – 4079 ng/g of bile) about three times higher than those from Gernika (283 – 1616
31 ng/g), although the variation between the three campaigns makes difficult to draw
32 definite conclusions (Figure 3A). The general tendency for both sites was a decrease in
33 OH-PAHs along the sampling seasons, however this decrease was more pronounced in
34 Gernika, where the lowest levels of OH-PAHs were observed in April 2008 (Figure
35 3A). Metabolite profiles differed in both sites, suggesting different sources of PAHs.
36 For example, 2-naphthol was the dominant PAH metabolite detected (38 – 71%) in fish
37 from Gernika, whereas in fish from Arriluze the major metabolite detected was of 1-
38 pyrenol (52 – 92%) (Figure 3B). 1-Pyrenol is considered a key PAH metabolite in fish
39 bile to monitor PAH exposure (Ruddock et al., 2002); consistently, its concentration in
40 bile was significantly higher in fish from Arriluze than from Urdaibai (Mann-Whitney
41 U test plus Bonferroni correction, $p=0.001$). Conversely, levels of 2-naphthol were
42 higher in Gernika than in Arriluze (Mann-Whitney U test plus Bonferroni correction,
43 $p<0.001$). Both 1-naphthol and 2-naphthol originate from naphthalene at very similar
44 ratios through a 1,2 epoxy intermediate (Varanasi et al., 1989; Yang et al., 1999), and
45 significant deviations on the concentrations of any of them are attributed to
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contamination from other sources unrelated to PAH metabolism, at least for human urine (Meeker et al., 2007; Serdar et al., 2003). Although it is possible that the ratios of synthesis and/or excretion of the two isoforms may differ between the two examined mullet populations, the almost 40-fold excess of 2-naphthol over 1-naphthol in the Gernika population (but not in the Arriluze population) argues for a specific contamination by some industrial process liberating 2-naphthol or a precursor substance. The fact that 2-naphthol made up to 70% of total biliar OH-PAHs in Gernika fish (Figure 3B) also argues for a specific source of this compound.

Biliary levels OH-PAHs in grey mullet were 3 to 45-fold higher than those detected in bile of *Trisopterus luscus*, a benthoplagic fish, collected along the inner shelf of the Basque Country coast (Fernandes et al., 2008a), suggesting a significantly higher anthropogenic pressure in the reserve of Urdaibai compared to the coastline. OH-PAHs levels found in grey mullet from Urdaibai were also similar or even higher than those detected in *Mullus barbatus* (91 – 1589 ng/g of bile) from the NW Mediterranean coast (Escartin and Porte, 1999). Direct comparison between different fish species is always difficult due to differences in habitat, metabolism and diet. This notwithstanding, the data suggest a significant contamination by PAHs in the Urdaibai endogenous fish population compared to the one observed in fish population from the coast, in line with our data on PAHs content and dioxin-like activity.

3.4 General discussion and conclusions

Sediments from Urdaibai showed a significant contamination by PAHs, particularly at the outer locality of Txatxarramendi. This site showed PAH levels comparable to those found in Arriluze, located in the neighbouring highly polluted Abra estuary. Blue mussels (*Mytilus galloprovincialis*) from Txatxarramendi show a high prevalence of oocyte atresia in developing or ripe bivalves (Puy-Azurmendi et al.), a condition that has experimentally been linked to the exposure to petroleum hydrocarbons (Ortiz-Zarragoitia and Cajaraville, 2006). These results were a first indication that levels of PAHs and other pollutants in Urdaibai may be exceeding tolerance levels for at least some animal species.

The origin of PAHs in Urdaibai and Arriluze seems to be mainly pyrolytic, with some contribution of petrogenic sources in Gernika, Murueta and Arriluze. Comparison between chemical data with predicted and observed dioxin-like activity values defined

1 different subsets of samples. Sampling points with strong marine influence, like
2 Mundaka and Laida, showed a low proportion of dioxin-like PAHs, although their total
3 PAH burden was similar to (or even higher than) that of other samples with much
4 stronger dioxin-like activity. Conversely, many samples from inside the estuary
5 presented dioxine-like activity values higher than predicted by their content in PAHs,
6 suggesting a contribution of dioxin-like compounds from other sources unrelated to
7 PAH pollution. Finally, relative concentrations of 1-pyrenol, 1-naphthol and 2-naphthol
8 in fish bile could indicate a specific industrial impact in Gernika relative to Arriluze,
9 which may be related to the excess of dioxin-like activity observed in the inner Urdaibai
10 samples. We conclude that PAHs are responsible for at least a substantial part of the
11 AhR ligand activity we observed in the AhR-RYA assay, consistently with their
12 property of being powerful AhR ligands. The substantial amounts of PAH metabolites
13 found in bile of fish from Gernika and Arriluze confirms recent exposure to PAHs,
14 although their actual physiological impact remains to be elucidated.
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Figure Legends

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4 Figure 1. Map of sampled sites. Sediment samples were collected at four localities
5 inside the Urdaibai estuary (Gernika, Murueta, Arteaga and Txatxarramendi, detailed
6 map on the right), Mundaka, at the river mouth, and Laida, outside the sand barrier
7 limiting the estuary (grey areas in the detailed map). The leisure harbour of Arriluze, in
8 the Abra estuary, was sampled as polluted external site. Fish were sampled only in
9 Gernika and Arriluze. The approximate outline of the main city in Urdaibai, Gernika, is
10 indicated with a discontinuous line at the bottom of the detailed map.
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18 Figure 2. A) Profiles of EPA PAHs contents (bars, left axis), AhR-ligand activity in
19 NFEQ (β -naphthoflavone equivalents, calculated by YCM-RYA; continuous line, right
20 axis) and predicted TEQ values (discontinuous line, arbitrary units) in sediment samples
21 from 6 localities in Urdaibai and from Arriluze, taken during three consecutive
22 campaigns. B) Value classification by K-means clustering (4 groups). Letters from "a"
23 to "d" indicate samples belonging to statistically differentiated clusters ordered by
24 increasing average (centroid) values; shadows of gray indicate the degree of pollution,
25 from the cleanest cluster (white, labeled "a") to the most contaminated one (black,
26 labeled "d").
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36 Figure 3. A) Biliary levels of hydroxylated-PAHs detected in thicklip grey mullets
37 collected in Gernika and Arriluze. Bars indicate total OH-PAHs content, SEM values
38 are represented by lines. Letters correspond to homogeneous subsets of data (ANOVA
39 plus Tukey's test, logarithmic transformants).
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45 B) Relative contributions of different hydroxylated metabolites to the total
46 hydroxylated-PAH contents in bile. Samples are identical to that of figure 3A.
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Table 1. Concentration (ng/g) of EPA PAHs and isomer ratios in sediments from the Urdaibai estuary

Area	Site	Sampling date	Naphthalene	Acenaphthylene	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo (a) anthracene	Benzo (b) fluoranthene	Benzo (k) fluoranthene	Benzo (a) pyrene	Chrysene	Indeno (1,2,3-c,d) Pyrene	Benzo (g,h,i) Pyrene	Dibenzo (a,h) anthracene	EPA PAHs	Isomer ratios ^a		
																				Ph/An	Fl/Pyr	BaA/Chr
Urdaibai, internal	Gernika	Apr-2007	42	1.1	2.7	2.9	22	4.3	11	18	46	58	65	72	45	20	19	4.5	435	5.07	0.63	1.02
		Oct-2007	214	2.0	4.1	5.0	152	58	47	78	232	294	340	296	283	60	61	4.7	2130	2.64	0.60	0.82
		Apr-2008	n.d.	305	3.1	322	264	79	13	34	449	552	346	345	n.d.	441	15	40	3209	3.33	0.39	
	Arteaga	Apr-2007	0.4	10	5.5	55	74	22	217	177	96	113	105	67	69	65	66	10	1153	3.32	1.22	1.39
		Oct-2007	0.5	13	6.9	8.5	111	33	137	149	187	208	240	149	167	99	100	12	1619	3.31	0.92	1.12
		Apr-2008	n.d.	154	4.5	155	156	57	42	103	318	384	173	166	118	502	122	301	2754	2.74	0.40	2.70
	Murueta	Apr-2007	66	805	38	6.7	62	19	18	22	114	133	114	71	74	66	68	10	1687	3.24	0.82	1.55
		Oct-2007	101	525	18	7.7	138	52	42	47	36	266	307	209	255	97	101	11	2213	2.64	0.90	0.14
		Apr-2008	304	181	3.3	145	237	92	38	110	424	528	165	232	35	712	68	98	3372	2.58	0.35	12.15
Urdaibai, external	Txatxarramendi	Apr-2007	102	615	11	126	611	227	1531	741	344	374	170	156	170	137	146	21	5482	2.69	2.07	2.03
		Oct-2007	236	966	17	1187	487	188	1020	557	451	467	244	226	248	1679	185	23	8182	2.58	1.83	1.82
		Apr-2008	n.d.	1649	90	497	696	284	1070	972	1048	1188	338	474	100	1716	226	258	10607	2.45	1.10	10.52
	Mundaka	Apr-2007	616	34	5.9	24	96	28	95	114	24	35	45	37	26	34	39	4.1	1257	3.42	0.84	0.95
		Oct-2007	962	9100	609	551	560	13	2075	1552	283	267	64	215	197	149	161	20	16778	42.35	1.34	1.44
		Apr-2008	32	19	4.4	11	22	7.9	52	38	42	57	17	11	8.7	2035	1312	925	4593	2.72	1.34	4.88
	Laida	Apr-2007	260	298	1286	125	10	45	160	160	41	44	53	42	31	35	36	6.4	2634	0.22	1.00	1.33
		Oct-2007	147	1286	362	185	30	82	115	114	177	143	192	147	134	43	53	11	3222	0.36	1.01	1.33
		Apr-2008	n.d.	11	2.9	13	14	4.1	17	17	27	37	23	30	n.d.	142	n.d.	n.d.	338	3.48	1.03	
Polluted reference	Arriluze	Apr-2007	1002	164	84	240	799	281	683	640	817	724	786	597	457	393	498	53	8217	2.85	1.07	1.79
		Oct-2007	230	246	88	215	593	221	50	84	766	675	1322	887	1032	285	401	35	7129	2.68	0.59	0.74
		Apr-2008	n.d.	523	145	578	907	300	22	59	1958	2626	2082	1195	n.d.	1135	n.d.	n.d.	11530	3.02	0.37	

a) Values diagnostic for pyrogenic origin of PAHs are marked in **bold**

n.d., not detected

Table 2. Spearman's rank correlation between AhR-ligand activity and concentration of PAHs in Urdaibai samples

	<i>Rho</i>	<i>Sigma</i> ^a	<i>n</i>
Naphthalene	-0.306	0.249	16
Acenaphthylene	0.268	0.241	21
Acenaphthene	0.134	0.563	21
Fluorene	0.269	0.239	21
Phenanthrene	0.647	0.002	*** 21
Anthracene	0.549	0.010	* 21
Fluoranthene	0.171	0.457	21
Pyrene	0.225	0.328	21
Benzo (a) anthracene	0.636	0.002	*** 21
Benzo (b) fluoranthene	0.622	0.003	*** 21
Benzo (k) fluoranthene	0.565	0.008	** 21
Benzo (a) pyrene	0.523	0.015	* 21
Chrysene	0.422	0.081	18
Indeno (1,2,3-c,d)pyrene	0.386	0.084	21
Benzo (g,h,i) perylene	0.426	0.069	19
Dibenzo (a,h) anthracene	0.263	0.276	19
EPA PAHs	0.342	0.130	21
TEQ	0.642	0.002	*** 21

a) Two-tailed significance level. *, $p < 0.05$; **, $p < .01$; ***, $p < 0.005$

Figure 1

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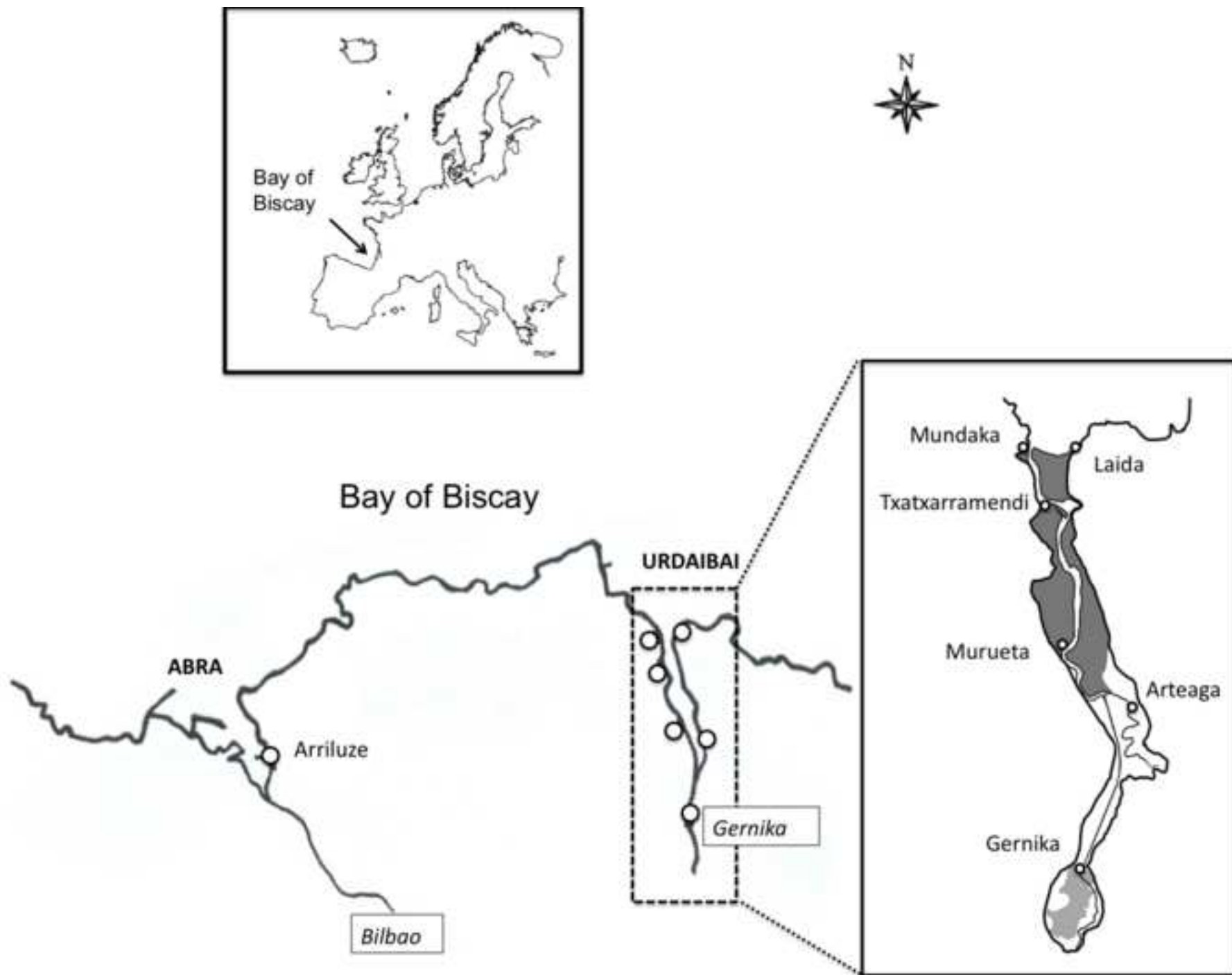
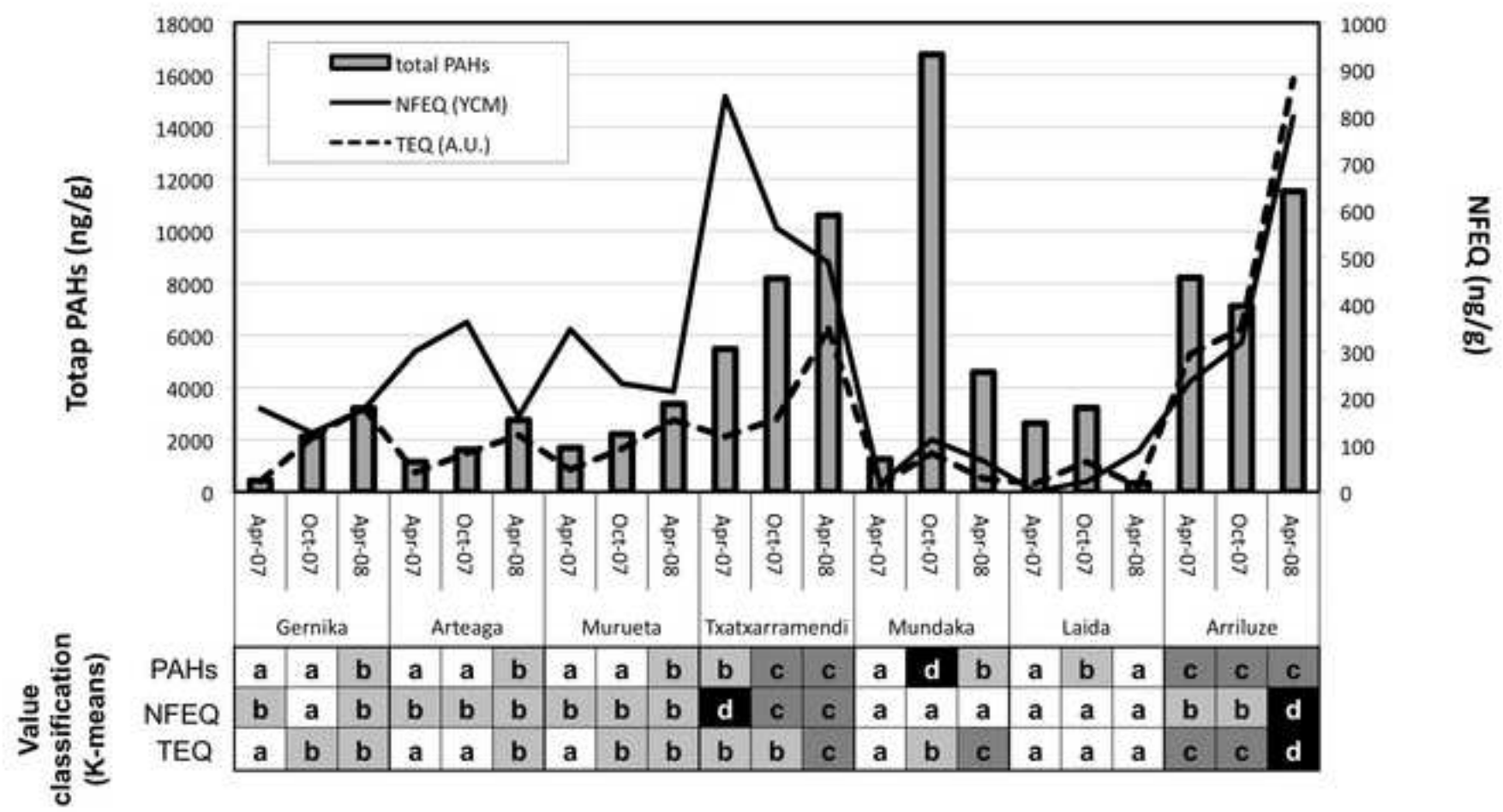
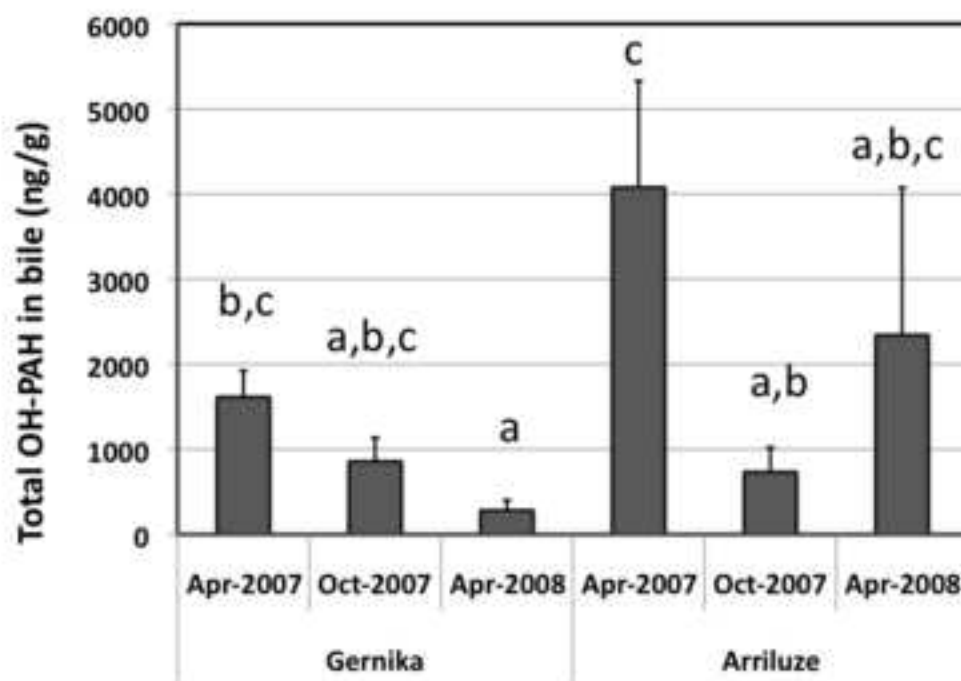


Figure 2

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A**B**