Feral finfish, and their relationships with sediments and seawater, as a tool for risk assessment of PAHs in chronically polluted environments

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HIGHLIGHTS

- The potential use of fish living in polluted areas as diagnosis tool has been studied.
- Concentrations of PAHs in three target organs of five species were determined.
- Histopathological effects, and correlations with abiotic compartments were studied.
- Potency Equivalent Concentrations and Screening Values were calculated and compared.
- Bioaccumulation factors and Toxic Potency Assessment of sediments were established.

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ABSTRACT

An integrated study has been carried out of the fate and effects of PAHs in fish living in a chronically polluted environment. Total PAH concentrations in different target organs (muscle, liver and gills), have been determined in five species of feral fish and possible histopathological effects and correlations of all these values with concentrations found in sediments and water column have been studied in two of these species. The Potency Equivalent Concentrations and Screening values (SVs), the Biota-to-sediment accumulation factors (BSAFs) and the Toxic Potency Assessment (TEQ) of sediment for fish have been calculated. Results show that levels found in target organs, the TEQs of sediment calculated for fish related to the SVs for fish consumption are of special concern. However, the index of pathologies shows a relatively low impact of PAHs on fish health. The use of feral finfish in risk assessment for PAHs in chronically polluted environments has been proved to be a useful tool to complement environmental diagnoses and improve their accuracy. This approach combines the measurement of total concentrations in different target organs of several appropriate species, the study of histopathological effects, and correlations between all these results and the concentrations found in associated sediments and column water.

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1. Introduction

Near-shore coastal areas such as estuaries are typically subject to a variety of stressors, both natural and anthropogenic, which can impair the health and fitness of resident biota (Salazar-Coria et al., 2010). To be able to ensure healthy, safe and prosperous oceans for the benefit of current and future generations, an ecosystem-based management is need to support sustainable developments, while taking into account the Precautionary Principle (Hellou et al., 2006; Council Directive, 2008/56/EC, 2008). Polycyclic aromatic hydrocarbons (PAHs) are a group of pollutants containing two or more fused benzene rings that are found widely in many environmental compartments.

The main sources of PAHs reaching the aquatic environment include the incomplete combustion at higher temperatures of fossil fuels and other more recent organic matter (pyrolytic sources) and the slow maturation of organic matter under conditions of geochemical gradient (petrogenic sources, including oil-spills) (Neff, 1979; McElroy et al., 1989; Rojo-Nieto et al., 2013). In coastal areas, anthropogenic PAHs enter the environment via run-off, industrial wastewaters and atmospheric emissions, vehicle exhaust gasses, and spillage of fossil fuels. Concentrations of PAHs in seawater and sediments are of toxicological significance to both benthic and pelagic marine organisms (Arias et al., 2009). As PAH solubility decreases with increasing molecular weight, organisms often become enriched in the lower molecular weight PAHs, relative to the sediment (Djomo et al., 1996; Porte and Albaigés, 1993), due to the higher bioavailability of these lower molecular weight PAHs because of their greater solubility/mobility from sediments. This is because the heavier molecular weight compounds are

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preferentially adsorbed on sediment or associated with particulate matter while the lower molecular weight PAHs are dissolved slightly more than the heavier PAHs, although all of them are hydrophobic (Baumard et al., 1999).

Some of these PAHs are chemicals with known carcinogenic potential (IARC, 2010); being them, and their reactive metabolites, of special concern, due to their environmental and human health effects (Mumtaz and George, 1995). Due to these properties and environmental implications, eight PAHs were included in the list of Priority Pollutants of the European Water Framework Directive (Council Directive, 2008/105/EC, 2008) and 16 in the United States Environmental Protection Agency (USEPA) Priority Pollutant List (Code of Federal Regulation, 1982). In the marine environment and more specifically in the European Southwest area there are two conventions of special relevance for Hydrophobic Organic Compounds (HOCs): Barcelona Convention and Oslo-Paris Convention (OSPAR). The Barcelona Convention, Convention for the Protection of the Mediterranean Sea against Pollution (1976, revised in 1995) defines some of the HOCs as Chemical Pollutants and together with the Mediterranean Action Plan are parts of the Regional Seas Program of the UNEP. The Oslo-Paris Convention, OSPAR (1992), through the OSPAR Commission (Protecting and Conserving the North-East Atlantic and its Resources), has the objective of prevention and removal of the pollution as well as the protection of the marine environment of the NE Atlantic from human activities. This convention includes the PAHs in the list of Chemicals for Priority Action.

Organisms living in environments contaminated with PAHs incorporate these compounds through their body surface and gills by sorption, or by ingestion of polluted sediment or suspended solids, transferring these pollutants through the aquatic food web (Patroilecco et al., 2010). Fish are considered top consumers with a relatively long life span and high biomagnification level of pollutants (Fang et al., 2009); consequently physiological impairment in fish is considered representative of general pollution in an aquatic system (Bervoets et al., 2009). However, according to Wan et al. (2007) PAHs undergo trophic dilution in the marine food web, which is likely to be the combined results of low assimilation efficiencies and efficient metabolic transformation at higher trophic levels. Increasing emphasis has been placed on the evaluation of the causal relationships between contaminant exposure and observable biological effects in aquatic organisms (De Flora et al., 1991). Fish pathologies are increasingly being used as indicators of environmental contamination, since they provide a definitive biological end-point of historical exposure (Matthiessen et al., 1993; Oliva et al., 2012). Several liver and gills lesions in marine fish have been shown to be associated with exposure to xenobiotic chemicals: these include neoplasms, non-neoplastic proliferative lesions and specific degenerative/necrotic lesions that are well established as histological biomarkers of response to contaminant exposure (Adams et al., 2003). Several authors have found histopathological effects produced in fish by PAHs, such as hydropic vacuolization, hepatocystic hypertrophy and liver tumors (Stentiford et al., 2003; Giacalone et al., 2004).

Given this worrying situation, the fate and effect of PAHs in biota are of special concern. This topic has been studied in the Bay of Algeciras (Spain), part of a unique and important coastal environment, located in South-western Europe, at the boundary between the Atlantic Ocean and the Mediterranean Sea. Due to this singular and special location, the Bay of Algeciras is contemplated into the boundaries of the Barcelona Convention and into the OSPAR Convention. This Bay has long suffered chronic anthropogenic pressure from urban and industrial activities (including a major petrochemical complex) and intense maritime traffic; the Port of Algeciras is ranked among the most important in the world (AAPA, 2007). The aim of this work is to improve tools for use in integrated studies of the presence, concentrations and effects of PAHs in species of fish. We study the concentrations in different target organs of several species, and their relationships with corresponding concentrations found in abiotic compartments (sediment and water column), together with the histopathological alterations observed in fish.

2. Material and methods

2.1. Sampling area

The Bay of Algeciras (36°7'51.91"N, 5°23'45.63"W) is located in the south of Spain, on the Strait of Gibraltar (Fig. 1). The Bay is 9 km wide and 10 km long, covering an approximate surface area of 70 km², with a depth of up to 400 m in the center of the bay and 460 m in the mouth. It is delimited by Punta del Carnero (near the city of Algeciras) in the west and Punta Europa (Gibraltar) in the east (Fig. 1).

This coastal area has suffered chronic anthropogenic impact for several decades from urban and industrial sources. The Bay of Algeciras is an important industrial area of the Mediterranean Sea, with a large refinery (capable of processing twelve million tons of crude oil annually) and its associated petrochemical industry, in addition to steel, paper and power production (4 thermal power plants). Another significant source of pollution is the intense maritime traffic associated with the transportation of oil to and from the refinery, bunkering activities in the Bay and the commercial shipping activities of the Port of Algeciras, one of the largest in Europe, which handled 43 million tons of goods in 2007. Five large population clusters are located around the Bay: the cities of Algeciras, Los Barrios, San Roque, La Línea de la Concepción, and Gibraltar. Together, these urban areas comprise more than 250,000 inhabitants (a population 'agglomeration' according to EU Directive 96/62/CE (Council Directive, 1996)). The water of the Bay has a high rate of turnover because of its proximity to the Strait of Gibraltar and the strong currents that predominate in this area. Despite these hydrodynamic conditions that promote dispersion of pollutants, due to the high concentration of sources of pollutants in the area (Fig. 1), studies of the anthropogenic effect on marine organisms in this Bay should have a high priority.

In this study, five sampling points have been selected as representative zones of the Bay (Fig. 1): "Getares" (point 1 in the figure), relatively distant from potential sources of PAHs; "Isla Verde" (2), an area influenced by untreated urban waste-water from Algeciras city (around 100,000 inhabitants); and "Palmones" (3), "Guadarranque" (4) and "Puente Mayorga" (5), points close to different industrial areas (a coal-fired power station, a steel processing plant, and a refinery – petrochemical complex, respectively).

2.2. Sample collection

Four sampling campaigns were carried out, in November '07 (SC1), May '08 (SC2), November '08 (SC3) and May '09 (SC4); at a total of 20 sampling stations, in the 5 selected locations, fish were caught during the four successive sampling campaigns (Fig. 1). Trammel nets of 500 m length, anchored during 24 h, were used for fishing. Fish caught were maintained alive in aerated seawater containers during their brief transport to the laboratory (less than 30 min), where they were sacrificed and dissected. Samples were obtained from the target organs selected (gills, muscle and liver). These tissue samples were divided in four portions: one for analysis of PAHs (stored in liquid nitrogen at –80 °C); a second portion for histopathological analysis (conserved in formalin 10%); and the third and fourth for analysis of other pollutants outside the objectives of this work.

2.3. Pooling

As the samples were obtained as a part of a large study in which several different pollutants were analyzed and different research groups were involved, it was necessary to share samples. Since the total volume of samples was not sufficient for the analytical methods proposed, it was necessary to group samples in pools to ensure the minimum mass necessary to carry out the PAHs analyses. As stated by Hellou et al. (2006), samples of similar morphometrics allow better interpretation; therefore, for these pools only individuals from the same location,

Fig. 1. Sampling area, showing the various sampling points and the most important industries, docks and ports. Sampling points: 1 "Getares", 2 "Isla Verde", 3 "Palmones", 4 "Guadarranque" and 5 "Puente Mayorga". * Coal-fired power station.

sampling campaign, species, and of similar weight and length were selected. 264 individuals were captured, but only 50 satisfied the requirements to be included in pools (Table 1).

2.4. Morphological index

The fish condition factor (CF) is one of the most used morphological indexes. It was calculated as the relationship between length and body weight using the following equation (Fulton, 1902):

$$CF = (W/L^b) * 100 \quad (1)$$

where W is the total body weight (g) and L is the fork length (cm), and b is assumed equal to 3 in *Solea* sp, *Trigla lucerna* and several other fish species with isometric growth (Jobling, 1994; Ricker, 1975; Fang et al., 2009; Abowei et al., 2009; Nash Richard et al., 2006; Stergiou and Moutopoulos, 2011).

2.5. Analytical procedure for PAHs

Samples were freeze-dried in a lyophilizer, grinded and homogenized. The procedures for PAHs analyses were based on the analytical method 3564C and 3620B proposed by USEPA (1996, 2000a), which

Table 1
Species studied.

Common name	Scientific name	Habitat	Feeding	Reference
Senegal sole	<i>Solea senegalensis</i>	Benthonic	Benthonic invertebrates, molluscs and crustaceans	Kaup, 1858
Tub gurnard	<i>Trigla lucerna</i>	Demersal	Fish, crustaceans and molluscs	Linnaeus, 1758
Red scorpionfish	<i>Scorpaena scrofa</i>	Demersal	Fish, crustaceans and molluscs	Linnaeus, 1758
Blackspot seabream	<i>Pagellus bogaraveo</i>	Bentho-pelagic	Omnivorous	Brünnich, 1768
Common two-banded seabream	<i>Diplodus vulgaris</i>	Bentho-pelagic	Crustaceans, worms and molluscs	Geoffroy Saint-Hilaire, 1817

according to Cheung et al (2007) are appropriate and optimum for analyzing PAHs in fish samples. Briefly, the samples were Soxhlet-extracted using a mixture of acetone and dichloromethane for 24 h; the extracts were purified on Florisil columns; and concentrated by rotary evaporation (Rojo-Nieto et al., 2011). The composition and concentration of 16 different PAHs were determined by gas chromatography-mass spectrometry (GC-MS/MS). A reference material (soil LGC6182) was analyzed, with 81–102 % of certified values of PAHs obtained, excepting Benzo(g,h,i)perylene. Average relative percent difference between triplicates (14%) was within acceptable limits, according to Levengood and Schaeffer (2011).

Details of concentrations in seawater and sediments from these sampling campaigns may be found in Rojo-Nieto et al., 2013, and in the supplementary data.

2.6. Calculation procedure to obtain Screening values for consumption of fish and Potency equivalent concentrations

The screening value (SV), for consumption of fish, is defined as the concentration of chemical in edible tissue that is of potential concern for public health. Screening values for PAHs in fish, according to the USEPA (2000b), can be calculated using the equation for carcinogenic compounds:

$$SV = [(RL/CSF) * BW]/CR \quad (2)$$

where RL is the risk level (and it is considered that the increased risk would be one additional cancer death at most per 100000 persons); BW is the body weight of the population (70 Kg being chosen for the average male adult); CR is the consumption rate (with two kinds of population being considered: subsistence fishers (fish consumption: 142.4 g/d) and recreational fishers (17.5 g/d)).

CSF is the Oral Cancer Slope Factor and it is not defined for all PAHs in the EPA Integrated Risk Information System (IRIS). In fact, it is only defined for Benzo(a)Pyrene (B(a)P), with a value of 7.3 mg/Kg/d.

Using Eq. (2), the SV for B(a)P is 5.47×10^{-3} mg/Kg for recreational fishers and 6.73×10^{-4} mg/Kg for subsistence fishers.

In order to transform individual PAHs to an equivalent concentration of B(a)P, for purposes of comparison with the SVs obtained, the Potency Equivalent Concentration (PEC) (Nisbet and Rasmussen, 1992), has been used. The PEC has been calculated using the following equation (Nisbet and Rasmussen, 1992):

$$PEC = \sum (RP_i * C_i) \quad (3)$$

RP_i being the relative potency for each PAH with respect to B(a)P, and C_i the concentration of each PAH.

Therefore, according to the Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories of USEPA (2000b), the PEC of total PAHs was calculated for each sample using Eq. (3), and compared with the Screening Value of Benzo(a)Pyrene (B(a)P), calculated using Eq. (2).

2.7. Histopathological study

The tissue samples for the histological examination were fixed in formalin (10%), washed in running tap water, dehydrated in alcohol and acetone, cleared in xylene and embedded in paraffin wax. Sections (6 μ m) were cut and mounted on gelatinized slides using a rotary microtome. Sections were rehydrated in distilled water and stained with hematoxylin/eosin (H/E) and hematoxylin/VOF (H/V) (light green, orange-G and acid fuchsin). Pathology criteria were derived from several tissue alterations specified by the International Council for the Exploration of the Sea (ICES) (Anon, 1999) and additional pathology observed in the sampled specimens. Histopathological sections were read at the end of each sampling period. Alterations were evaluated

semi-quantitatively by ranking the severity of the alteration in the tissue. Ranking was: grade 0 (no alterations), grade 1 (focal mild alteration), grade 2 (moderate alteration) and grade 3 (extended severe alteration). This ranking was used to establish an overall assessment value of the histopathological lesions for each organ of individual fish.

2.8. IPAT (Index of Pathologies)

IPAT is an index modified from the histopathological condition index of Bernet et al. (1999) (Oliva et al., 2012). For each alteration, an importance factor or condition weight (w) was assigned, as proposed by Bernet et al. (1999), based on the biological significance of the lesion, i.e. the grade at which a lesion may affect the normal functioning of a tissue or organ. The alterations are classified into three importance factors (condition weights): (1) Minimal pathological importance, the lesion is easily reversible when exposure to pollutants ends; (2) Moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized; (3) Marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function. In this work, the prevalence of lesions has also been included in the index calculation because IPAT is not an organ pathology index but rather a general index for the pathological state of fish from a specific site and season. The index was obtained for each sampling site and campaign and was calculated using the equation:

$$IPAT = \sum_{i=1}^n \frac{alg_i \times p_i \times w_i}{100} \quad (4)$$

where alg is the average lesion grade of the alteration, p is the prevalence of this alteration, w is the condition weight and n is the number of pathologies analyzed in each fish.

For each site and sampling IPAT was calculated to establish an assessment value varying from 1 to 54. Assessment values ≤ 17 were assumed to constitute a low impact on fish health, values between 18 and 36 were assumed to constitute a moderate impact, and values > 36 were assumed to constitute a high impact on health fish.

2.9. Statistical analysis for biological data

Statistical analyses were performed using the statistical software package STATISTICA 6.0 (data analysis software system) (2004, Statsoft, Inc. USA). For each data set, the assumptions of an analysis of variance (ANOVA), normality and equal variance, were checked using Shapiro-Wilks and Levene tests, respectively. If the assumptions were not met, the necessary transformations were performed. If a data set has a normal distribution, one-way ANOVA were conducted and where significant, a post-hoc mean comparison test (Tukey's test) was conducted (parametrics). If a non-normal data set was found, the Kruskal-Wallis and Mann-Whitney (one-tail) tests to find significant differences were conducted (non-parametric). To analyze the correlation between alterations and concentrations of pollutants, the Pearson (parametric) and Spearman (non-parametric) correlations test were used. All statistical analyses were conducted at an alpha level of 0.05.

3. Results and discussion

3.1. Presence and levels of PAHs in fish

All analyzed samples, with the exception of 3 samples from the same pool (gills, liver and muscle from Pool 1), showed the presence of PAHs (Table 2). Generally, the main PAHs found were Phenanthrene and Pyrene; Naphthalene and Fluorene were also detected, but frequently under the quantification limit, and so were not included in the sum of PAHs. Total concentration of PAHs ranged between 'none detected' and 358.44 μ g/Kg. According to Ribeiro et al. (2005), Pyrene is frequently used as a marker of the total PAHs contamination in fish, with

Table 2
Concentrations of PAHs in target organs ($\mu\text{g/Kg}$ d.w.), condition factor (CF), Potency Equivalent Concentrations (PECs, $\mu\text{g/Kg}$) and Biota-to Sediment Accumulation Factors (BSAFs).

Pool	$\Sigma 16$ PAHs Gills	$\Sigma 16$ PAHs Muscle	$\Sigma 16$ PAHs Liver	Average CF	Species	Sampling Campaign	Station	PEC Gills	PEC Muscle	PEC Liver	BSAF Gills	BSAF Muscle	BSAF Liver	Average BSAF
P1	<d.l.	<d.l.	<d.l.	0.90	<i>S. senegalensis</i>	SC2	P2							
P2	325.37	187.55	358.44	0.93	<i>S. senegalensis</i>	SC2	P1	0.325	0.188	0.358	1.713	0.987	1.887	1.529
P3	188.56	73.57	226.13	0.99	<i>S. senegalensis</i>	SC2	P1	0.189	0.074	0.226	0.993	0.387	1.191	0.857
P4	184.07	107.59	151.14	0.99	<i>S. senegalensis</i>	SC2	P1	0.184	0.108	0.151	0.969	0.566	0.796	0.777
P5	131.50	78.42	102.18	0.92	<i>S. senegalensis</i>	SC2	P1	0.131	0.078	0.102	0.692	0.413	0.538	0.548
P6	114.34	77.32	195.33	0.79	<i>T. lucerna</i>	SC2	P3	0.114	0.077	0.195	0.203	0.137	0.347	0.229
P7	205.49	82.74	385.25	0.91	<i>T. lucerna</i>	SC2	P3	0.205	0.083	0.385	0.365	0.147	0.684	0.399
P8	293.26	145.17	176.34	0.91	<i>T. lucerna</i>	SC2	P3	0.293	0.145	0.176	0.521	0.258	0.313	0.364
P9	118.61	108.96	241.00	0.77	<i>T. lucerna</i>	SC2	P3	0.119	0.109	0.241	0.211	0.194	0.428	0.277
P10	167.40	93.61	89.96	0.95	<i>S. senegalensis</i>	SC2	P5	0.167	0.094	0.090	1.238	0.692	0.665	0.865
P11	81.89	64.07	285.16	1.01	<i>S. senegalensis</i>	SC1	P1	0.082	0.064	0.285	0.072	0.056	0.250	0.126
P12	28.78	18.73	33.49	0.91	<i>S. senegalensis</i>	SC4	P1	0.029	0.019	0.033	1.246	0.811	1.450	1.169
P13		29.64	26.88	1.07	<i>S. senegalensis</i>	SC4	P1		0.030	0.027		1.283	1.163	1.223
P14	76.08	19.15	141.77	2.16	<i>T. lucerna</i>	SC4	P4	0.076	0.019	0.142	0.053	0.013	0.099	0.055
P15	111.16	19.89	109.04	1.37	<i>P. bogaraveo</i>	SC4	P4	0.111	0.020	0.109	0.078	0.014	0.076	0.056
P16	216.81	51.77	174.46		<i>D. vulgaris</i>	SC4	P2	0.217	0.052	0.174	3.219	0.769	2.590	2.193
Average	160.24	77.21	179.77								0.827	0.449	0.832	

<d.l. Below detection limit.

Naphthalene and Phenanthrene also being relatively abundant in fish. The major presence of these PAHs found in this study could be due to the combination of two factors. It can be explained, first, by the specific characteristics of the study area with respect to distribution of PAHs; and, second, by the high degree of biotransformation of some PAHs in fish.

Concerning the study area, the presence of these four PAHs is in accordance with concentrations and distribution of PAHs observed in sediments collected from the same points at the same times as the fish (Rojo-Nieto et al., 2013). In that study, Phenanthrene (Phen), Pyrene (Pyr) and Naphthalene (Naph) were three of the four PAHs which most frequently showed higher concentrations in the sediment samples. Moreover, an environmental diagnosis carried out in the atmosphere of the study area in 2004 (CSIC, 2004), found that three of the four main PAHs present in the atmosphere were Pyr, Phen and Fluorene (Flu), but Naph was not analyzed. Another study of PAHs in soils sampled from land surrounding Algeciras Bay indicated that main PAHs present, by atmospheric deposition, were Phen and Pyr (Posada-Baquero, 2007). It is also interesting that, as the solubility of PAHs decreases with increasing molecular weight, the bioaccumulation of PAHs by marine organisms from sediments is generally greater for the lower molecular weight and more water-soluble PAHs (Porte and Albaigés, 1993; Djomo et al., 1996; Baumard et al., 1998). All these results indicate that the major presence of these PAHs in fish is influenced, in part, by the sources of these chemicals in the studied area, due to the four main PAHs detected in fish are the same four main PAHs present in other environmental compartments of the zone (atmosphere, soil and marine sediments).

Concerning biotransformation, fish do not accumulate large amounts of PAHs because of their reduced uptake and/or increased clearance of these compounds (Patrolecco et al., 2010; van der Oost et al., 2003). Despite this, chronic exposure (as in the case of this study) does lead to the accumulation of PAHs in fish, from sediment and/or food (Arnot and Gobas, 2006; Farrington, 1991; Simcik and Offenberger, 2006). Fish, especially benthic species like flatfish, are used as indicators of pollution of sediment by PAHs (Levengood and Schaeffer, 2011). The more advanced fish families possess the means, in the Ah receptor, to metabolize PAHs, and therefore are not so prone to accumulation (Arnot and Gobas, 2006; Hahn et al., 1994; Levengood and Schaeffer, 2011). High molecular-weight PAHs are metabolized and excreted more rapidly than lower molecular-weight PAHs, possibly accounting for the higher concentrations of low molecular-weight PAHs sometimes found in fish tissues (Varanasi and Stein, 1991; Hellou et al., 1995, 2006; Baumard et al., 1998; Neff, 2002).

This high biotransformation in fish may be linked with the condition factor (CF), which can be understood as a general measure of the nutritional status: it is assumed that a heavier fish of a given length is in better condition. Low CF values (<1 for *Solea senegalensis*) could indicate fish fitness under stress of pollution, because a metabolic trade-off is required to deal with detoxification and the energy available for growth may thus be reduced (Fang et al., 2009). The CF in fish was reported to decline on exposure to pollutants, including PAHs (Khan, 2003; Fang et al., 2009). In this study, the CFs obtained are low, and in 70% of the cases the CF is below 1 (Table 2). Van den Heuvel et al. (2008) suggested that a detectable change in the physiological endpoints, such as the CF, might take at least two years to reflect the altered energy intakes in fish due to the effect of pollution. Therefore, levels of PAHs found in the target organs (Table 2), together with the fact that fish metabolize PAHs, seem to indicate that these fish are subjected to a long-term chronic exposure, could the observed values of CF be a result which supports this assumption (Tables S1 and 2). Non-detectable levels of large PAHs in fish tissues could indicate a more efficient metabolism and a higher potential toxicity (because metabolites are frequently more toxic than parent compounds), especially if there is chronic exposure (Hellou et al., 2002; Hellou and Leonard, 2004). In the case of this study, large PAHs have been detected in all sediment and water samples (Rojo-Nieto et al., 2013 and supplementary data), but not in fish.

Considering the levels of total PAHs obtained in fish target organs (Table 2), there is no clear difference between the only two species for which more than three pools of tissue were analyzed, *S. senegalensis* and *T. lucerna*. On the other hand, there are differences between organs: concentration in muscle tissue is about half that found in gills and liver. As can be observed in Table 2, the average PAH concentration was $77.21 \pm 22.79 \mu\text{g/Kg}$, $160.24 \pm 39.37 \mu\text{g/Kg}$ and $179.77 \pm 50.43 \mu\text{g/Kg}$ in muscle, gills and liver respectively. This is consistent with the findings of Neff (2002), who states that concentrations in fish can be higher in liver than in muscle. With the pools analyzed, spatial or temporal differences could not be stated. However, during sampling campaigns, it was realized that, at sampling point 5, the number of captures were significantly lower than in the rest; the total number of individuals captured there in each sampling campaign was between 0 and 7, while at the rest of the sampling points, numbers captured ranged between 10 and 50.

3.2. Biota-to-sediment accumulation factors

Because they can accumulate in sediments, PAHs in this compartment have often been examined in relation to the role played in exposure and

effects in aquatic life. PAH compounds are considered to be mutagenic and carcinogenic, and, consequently, their bioaccumulation is relevant and of special interest because it provides information about long-term toxicity (Milinkovitch et al., 2011; Ramachandran et al., 2004). Furthermore, even without detectable acute or chronic effects, bioaccumulation should be regarded as a hazard criterion in itself, since some effects may only be recognized in a later phase of life (Van der Oost et al., 2003). According to several authors (Baumard et al., 1998; Hellou et al., 2006; Cheung et al., 2007; Patrolecco et al., 2010) Biota-to-sediment accumulation Factors (BSAFs) can be calculated as the relationship between the concentration in the organism and the concentration in the sediment with which this organism is associated, as habitat or food source. In this study, BSAFs have been calculated for gills, liver and muscle from each pool (Table 2). Differences have been found between organs (Table 2), with BSAF in gills (average 0.83 ± 0.45) and liver (average 0.83 ± 0.36) being double that in muscle (average 0.44 ± 0.20). On one hand, according to Liang et al. (2007), the majority bioaccumulation of Low-Molecular-Weight-PAHs in the tissues suggested that bioavailability of relatively low K_{ow} isomers is high due to higher gill transfer efficiencies (aqueous uptake) in fish and decreased gut assimilation (dietary uptake) results in decreased accumulation of more hydrophobic PAHs (high K_{ow}). On the other hand, according to Neff (2002), there is growing evidence that the route of uptake of PAHs may have a profound effect on their distribution and persistence in the tissues of the animal: little or none absorbed from the gut accumulates in muscle and other tissues of fish, whereas PAHs that are taken in through the gills are transported in the general circulation throughout the body and can accumulate in several types of tissue, particularly well-perfused tissues rich in lipids. Taking all previously exposed into account, and considering both the compounds and the levels found in target organs, this study confirms the relationship between levels found in fish and in the surrounding environment, with PAHs being incorporated mainly through gills and accumulated in lipid tissues, like liver.

BSAFs of individual compounds can be used for inter-species comparisons, according to Baumard et al. (1998). In this study, BSAFs of Phen and Pyr were used for this objective (Fig. 2). Only those species for which more than three pools (of all organs) were available have been studied, i. e. *S. senegalensis* and *T. lucerna*. In the target organs considered, it can be observed (Fig. 2) that, in these two species, biota-to sediment accumulation factors are completely different: in *S. senegalensis* the BSAF is >1 (i.e., effective concentration from the environment exists); and in *T. lucerna* the BSAF is <1 . Average BSAF values for all pools and organs are shown in Fig. 2b. The value for Phen is 4.46 ± 0.29 and 0.52 ± 0.29 in *S. senegalensis* and *T. lucerna* respectively; in the case of Pyrene, these values are 5.53 ± 0.41 and 1.06 ± 0.58 for *S. senegalensis* and *T. lucerna*.

Fig. 2a shows individual BSAFs in the three organs, in these two species, with the 95% confidence interval indicated. In this graph, it can be observed that, with no exception, individual BSAFs obtained for different target organs are higher in *S. senegalensis* than in *T. lucerna*. In Fig. 2a and b it can be observed clearly that there are significant differences between the BSAFs of individual PAHs in these species. These differences found for the BSAFs of Pyr and Phen in these species could be due to differences in the incorporation rate of the pollutants and/or in the capacity for biotransformation of the PAHs. In fact, although a bioaccumulation ratio of <1 could be interpreted, in general, as a non-bioaccumulation process, in the case of fish, it could actually be due to a high biotransformation rate, as was asserted by Mayes and Barron (1991a,b). According to these authors, since the process of sorption and bioaccumulation are both governed by the hydrophobicity of the chemicals involved, in the case of fish, degradation and biotransformation are most likely responsible for the virtual absence of bioaccumulation of PAHs. The Pyrene Fish Biotransformation rate can be qualitatively described as Moderate, according to Burkhard et al. (2012a). Another caveat is that to calculate BSAF steady-state is required (Burkhard

Fig. 2. Individual BSAFs of Phenanthrene and Pyrene, with the 95 % confidence interval indicated. a) In various target organs. b) Average for each PAH in each species.

et al., 2012b), and field data cannot be fully ensure this equilibrium state. Despite these limitations, BSAFs have been proposed as a simple screening tool to estimate bioaccumulation in fish, as the first tier of a multi-tier evaluation (Wong et al., 2001; Van der Oost et al., 2003).

Presented in Table S2 (in Supplementary data) are biota-to-sediment accumulation factors for adults of *S. senegalensis* captured during the spawning period. For these, Phen and Pyr concentrations in muscle and liver from campaigns SC2 and SC4 (coinciding with spawning period) were selected, and the medium lipid content in muscle and liver in this reproductive period from Norambuena et al., 2012 was used, being, the specimens, adults of exactly the same size and species.

3.3. Potency equivalent concentrations

Using Eq. (2), the SV for B(a)P is 5.47×10^{-3} mg/Kg for recreational fishers and 6.73×10^{-4} mg/Kg for subsistence fishers. The Potency equivalent factors (PEC) values calculated are presented in Table 2. As can be observed in the table, no PECs for muscle, liver or gills have values higher than the Screening values (SVs) proposed by the USEPA for B(a)P for either recreational or subsistence fishers. These results are in agreement with those obtained in a study of fish from Hong-Kong market (Cheung et al., 2007). However, as observed in Fig. 3, some samples are close to the Screening Value for subsistence fishers, being of the same order of magnitude. Given that, the concentrations of PAHs found in fish from this study area could be of special concern. Moreover, according to the recommendations of the French Agency for Food Safety (AFSSA, 2003) for fish consumption, issued as a consequence of the 'Prestige' oil spill disaster which happened in 2002, the total concentration of 16 individual PAHs should not be above 50 µg/Kg. In the case of this study, 69% of the muscle tissue samples exceed this value. While the USEPA (2000b) guidance applies a conversion of concentration of different PAHs according to their different carcinogenic potential (transforming these to B(a)P equivalent

concentrations), the AFSSA recommendation takes into account the sum of the 16 priority PAHs, without making any distinction between them. One feasible explanation for this difference between the two criteria (EPA and AFSSA) in muscle is that the PAHs found in fish muscle are not the most carcinogenic (mainly low molecular weight PAHs).

3.4. Toxic Potency Assessment of sediment for fish

Since for some sampling points there were no data on PAHs in fish or no histopathology data, an approach that links fish and sediment was carried out. In order to estimate the potential toxicity for fish of PAHs found in sediments, some authors (Salazar-Coria et al. (2010) and Colombo et al. (2006)) have used the Dioxin-Equivalent Toxicity Factors (TEQs) of PAHs for fish (also named Toxicity Equivalent Concentrations).

TEQs were calculated for the 20 sediments sampled at the same time as the fish (Rojo-Nieto et al., 2013 and Table S5), by multiplying the concentration of individual PAHs determined in sediments by the Fish Potency Factors (FPFs) (Barron et al., 2004). These FPFs, which may be used to perform a PAH toxicity screening for fish, are derived from CYP1A induction and AhR binding, two biomarkers associated with PAHs in fish (Barron et al., 2004). The total TEQ for each sample was calculated as the sum of the individual TEQ of each PAH. The values of TEQs obtained are presented in Table S5. As can be observed, Total TEQs ranged between 3.10 pg/g and 376.37 pg/g. For 12 of the 20 sediment analyzed total TEQ values obtained exceed the suggested Safe Sediment Value of 20 pg/g (Eljarrat et al., 2001), and 9 of them are above the No Observed Effect Concentration (NOEC) of 200 pg/g. The Safe Sediment Value of 20 pg/g has been formulated for chronic exposure situations (Eljarrat et al., 2001).

3.5. Correlations between histopathological alterations and PAH concentrations in abiotic compartments

Seven major types of histopathological lesions were analyzed in gill tissues of *S. senegalensis*: aneurysms, hypertrophy of the lamellar epithelia, hyperplasia of the lamellar epithelia, hypertrophy of cartilage, lamellar fusion, desquamation of the lamellar epithelia and epithelial lifting. Gills from *S. senegalensis* showed an average high prevalence and lesion grade for aneurysms, desquamation and epithelial lifting (Table 3). Six major types of histopathological lesions were analyzed in liver: steatosis (fatty liver disease), vacuolization, necrosis, blood stagnation, leucocitary infiltration and hepatic parenchyma disorganization. Hepatic parenchyma disorganization showed an average high prevalence and lesion grade in liver (Table 3). Significant differences ($p \leq 0.05$) were observed in the prevalence of aneurysms between seasons (fish captured in spring presented high prevalence of aneurysm, in comparison with fish captured in autumn). No significant differences ($p \leq 0.05$) were observed in the lesion grade of alterations between sampling sites or seasons for *S. senegalensis*.

To study correlations between levels of PAHs found in sediment and water column and the different pathologies detected in fish, correlation matrices were carried out. Shown in Table 4 are the correlation coefficients (r) of the significant correlations ($p \leq 0.05$) between prevalence of alterations in *S. senegalensis* and concentrations of PAHs in sediments and water. Correlations have mainly been found with PAHs present in the water column. On the one hand, this is consistent with previous results obtained in the study of BSAFs (see section on *Biota-to-sediment accumulation factors*), where all seemed to indicate that PAHs were mainly incorporated through the gills. On the other hand, in this study, water column samples were analyzed without previous filtering; concentrations of PAHs determined in water show a close relationship with suspended particulate matter (SPM) with which PAHs are also associated, and through this, related to the sediment (Rojo-Nieto et al., 2013). Taking this into consideration, correlations with the column water imply, indirectly, also correlations with the sediment.

PAH concentrations in column water are correlated with three histopathology effects in liver and one in gills. In the latter, correlation has been found between the desquamation of the lamellar epithelia and the concentrations in column water of the PAHs with molecular weight 228 and 252. In the case of PAH concentrations in sediment, fewer correlations are found and these appear in pathologies of gills (not related to pathologies of liver), in the form of lamellar fusion, which is correlated with the concentrations of Naphthalene, Phenanthrene and Benzo(k) fluoranthene in sediments. Gills are extremely important in respiration, osmo-regulation, acid–base balance, and excretion of nitrogenous wastes in fish, and they account for the largest surface area of the animal in contact with external environment. Therefore, gill morphology can be very useful as a parameter in environmental monitoring (Schwaiger et al., 1997). In the present study, the gills of *S. senegalensis* showed the occurrence of histological alterations. Some of these alterations can be considered adaptive, since they protect the organism from taking in xenobiotics, as in the case of epithelial lifting. Aneurysm, on the other hand, is a lesion that can result from the rupture of pillar cells, and thus corresponds to the deleterious effect of xenobiotics on branchial tissue (Martinez et al., 2004). Several PAHs have been correlated with the desquamation of the lamellar epithelia in gills of *S. senegalensis*. In the gills of flounder (*Pleuronectes americanus*) captured near an oil refinery, Khan (1998) found hyperplasia in the lamellae and in the inter-lamellar space, as well as epithelial lifting, and this author suggested that the lesions found were related to oil spills. In another study with flounder captured at a location contaminated with PAHs, gills showed alterations such as hyperplasia and hypertrophy of the lamellar epithelium resulting in fusion and increase in mucus production (Khan, 2003). Results show that branchial lesions in *S. senegalensis* can compromise various other functions of the gills and thereby cause functional disturbances in this fish.

In the case of liver, hepatic parenchyma disorganization (HPD) appears to be correlated with the majority of PAHs of both types,

Fig. 3. Potency Equivalent Concentrations (PECs) in pools of different target organs and Screening Values (SVs).

Table 3

Prevalence (%) of pathologies recorded in the gills and liver of *S. senegalensis* captured in November 2007, May 2008, November 2008 and May 2009. A (aneurysms), HPT (Hypertrophy of the lamellar epithelia), HPL (Hyperplasia of the lamellar epithelia), HPC (Hypertrophy of cartilage), LF (Lamellar fusion), D (Desquamation of the lamellar epithelia), L (Epithelial lifting), E (Esteatosis), V (Vacuolization), N (Necrosis), BS (Blood stagnation), LI (Leucocitary infiltration), and HPD (Hepatic parenchyma disorganization).

Sites Collection date		Gill							Liver					
		A	HPT	HPL	HPC	LF	D	L	E	V	N	BS	LI	HPD
<i>Getares</i>														
n = 3	Nov '07	–	–	–	–	–	–	–	0	66.6	0	0	66.6	0
n = 15	May '08	46.6	0	60	0	46.6	33.3	53.3	0	13.3	0	13.3	6.6	26.6
n = 6	Nov '08	33.3	0	50	0	33.3	100	50	0	16.6	0	0	16.6	50
n = 7	May '09	42.8	28.5	85.7	0	28.5	71.4	28.5	57.1	14.2	28.5	42.8	42.8	100
<i>I. Verde</i>														
n = 3	May '08	33.3	0	66.6	0	33.3	33.3	66.6	0	0	0	0	0	0
<i>Palmones</i>														
n = 3	May '08	100	0	0	0	33.3	100	100	0	33.3	0	0	0	0
n = 4	Nov '08	25	0	50	0	0	100	100	0	75	0	75	25	100
<i>Guadarranque</i>														
n = 1	May '08	100	0	100	0	100	100	0	0	0	0	0	0	0
n = 2	May '09	100	0	50	0	0	100	0	50	0	0	50	50	100
<i>Mayorga</i>														
n = 1	Nov '07	–	–	–	–	–	–	–	0	0	0	100	0	0
n = 3	May '08	100	0	0	0	0	100	100	0	66.6	0	0	0	33.3
n = 3	Nov '08	0	66.6	0	0	33.3	100	100	0	66.6	0	33.3	0	66.6

those of low (LPAH) and high (HPAH) molecular weight (Table 4). In contrast, steatosis appears to be correlated with a specific group of PAHs, with those of molecular weight ≤ 202 (i.e. Fluoranthene, Pyrene

Table 4

Coefficients (r) of the significant correlations ($p \leq 0.05$) between alterations prevalence in *S. senegalensis* and PAH concentrations in sediments (S) and water (W). It has been studied 13 alterations: A (aneurysms), HPT (Hypertrophy of the lamellar epithelia), HPL (Hyperplasia of the lamellar epithelia), HPC (Hypertrophy of cartilage), LF (Lamellar fusion), D (Desquamation of the lamellar epithelia), L (Epithelial lifting), E (Esteatosis), V (Vacuolization), N (Necrosis), BS (Blood stagnation), LI (Leucocitary infiltration), and HPD (Hepatic parenchyma disorganization)), but in the table are only shown those which presented relevant correlation results.

		LF	D	E	BS	LI	HPD
Naphthalene	S	0.6462					0.8616
	W			0.6833			
Acenaphthylene	S						
	W			0.6408			0.9002
Fluorene	S						
	W			0.7570			
Phenanthrene	S			0.6607	0.6840		0.8784
	W						
Anthracene	S						
	W			0.6607	0.6840		0.8784
Fluoranthene	S						
	W			0.6622	0.7029		0.8921
Pyrene	S						
	W			0.6622	0.6806		0.8709
Benzo(a)anthracene	S						
	W						0.8224
Chrysene	S			0.7559			
	W			0.7245			0.8531
Benzo(b)fluoranthene	S						
	W			0.7559			0.8219
Benzo(k)fluoranthene	S	0.8944					
	W			0.7559			0.8224
Benzo(a)pyrene	S						0.8720
	W						
Indene(1,2,3, cd)pyrene	S					0.8451	
	W					0.9411	0.9258
LPAH	S						
	W			0.6396			0.8767
HPAH	S						
	W						0.7844
TPAH	S						
	W						0.8036

and LPAH); similarly, blood stagnation is correlated with PAHs of molecular weight 178 and 202 (i.e. with Phenanthrene, Anthracene, Fluoranthene and Pyrene). The liver is one of the most important organs for detoxification in the organism and can be considered a fat tissue since it generates and stores lipids. PAHs have a high K_{ow} , so they tend to accumulate in adipose tissues. The interaction between PAHs and lipids in tissues can prevent the normal functioning of mixed-function oxygenase (MFO). MFO is an enzymatic system that metabolizes organic compounds such as PAHs. The increase of PAHs in liver produces damage in the cellular lysosomal system that can result in a fatty degeneration. A similar effect was observed by Moore (1988) in digestive cells of *Mytilus edulis* and *Littorina littorea* exposed to PAHs and polychlorinated biphenyls (PCBs). Blood stagnation in liver has also been correlated with several PAHs analyzed in this study. This correlation can be explained through the action of δ -aminolevulinic acid dehydratase (ALAD), a biomarker of metabolic stress. The formation of ALA is a rate-limiting factor in the regulation of heme synthesis; as ALA increases, heme also increases. Under normal conditions, the heme synthesis pathway is regulated by a feedback mechanism which will inhibit the formation of ALA when heme reaches a threshold. However, when an organism is exposed to certain environmental contaminants, like PAHs, control mechanisms break down, resulting in an increased accumulation of ALA, which may cause increased levels of ALAD such as those observed in the study of Martin and Black (1996) in channel catfish exposed to ethyl benzene, toluene, xylene, 2-methylnaphthalene, phenanthrene and naphthalene in water.

No PAH has been correlated with the presence of vacuolization in the liver of *S. senegalensis* (at least in an individual form) although PAHs have been shown to be risk factors for hydropic vacuolization in fish (Johnson et al., 1992). Vacuolization is an alteration that contributes to liver parenchyma disorganization, and this alteration is correlated with the major PAHs analyzed in this study. Its occurrence in certain species has been strongly associated with exposure to hydrocarbons; and its utility as a histological biomarker for xenobiotic exposure, at least for those species, is established (Stehr et al., 1998; Ribeiro et al., 2005; Agamy, 2012). Liver parenchyma disorganization is also produced in some cases by the deformation of hepatocytes resulting from hyperplasia and hypertrophy phenomena. Hepatocyte hypertrophy with micro-vesicular formation is the most prominent lesion observed in Atlantic cod (*Gadus morhua*) exposed to water-accommodated oil fractions containing polycyclic aromatic hydrocarbons (PAHs) and in pearl

dace (*Margariscus margarita*) sampled at a diesel fuel-contaminated still-water pond (Khan, 1999).

An additional complicating factor in the interpretation of data for lesions is the offshore migration of *S. senegalensis* during the spring season. Although experimental evidence has shown that liver neoplasms can be induced by exposure of fish to contaminated sediments (Metcalf et al., 1988), the development of these lesions is thought to occur slowly. Furthermore, it has been suggested that regression and re-modeling of lesions, such as hepatocellular FCA, back into normal parenchyma, is possible if the stimulating contaminant is removed. During the offshore period, fish of this species are likely to be exposed to an array of potential contaminants different from those experienced in the home estuary. However, it should be noted that the dab (*Limanda limanda*), an offshore species of flatfish that is used in national monitoring programs of biological effects in the North Sea, exhibits a relatively high prevalence of hepatic alterations (CEFAS, 1998); this suggests that contaminant effects will also continue in coastal waters. Majority of analyzed fish are benthonic or demersal, with a practically sedentary life and a very territorial biology. For this reason, the contact between fish and polluted sediment of the study area is prolonged. Due to all of this, it is likely that any lesion detected in this species provides a sensitive measure of past and current contaminant exposure, and are thus extremely useful biomarkers of species, population and ecosystem health.

Other species studied in this work, particularly *T. lucerna*, are also suitable for histopathological study. Due to the low number of individuals of *T. lucerna* captured, our study has been centered on *S. senegalensis*. Nevertheless, in the case of *T. lucerna*, it has been studied for additional data to corroborate (or not) the findings for *S. senegalensis*. As in the case of *S. senegalensis*, in *T. lucerna* more correlations are observed with PAH concentrations in column water (with SPM included) than in sediments. However, since the amount of data available for statistical analysis is less, it is presented as supplementary material.

As a consequence of the results obtained in correlations between histopathology and concentrations in abiotic compartments, BCFs (bio-concentration factors from column water) of Phen and Pyr in fish have been calculated (given in the supplementary information), in addition to BSAFs in sediment reported in this study. Differences in bioaccumulation, similar to those observed for sediment, have been found between *S. senegalensis* and *T. lucerna*, but of a different order of magnitude, due to the solubility of PAHs in water.

4. Conclusions

In the study area, some levels of PAHs found in target organs of fish are below the SVs proposed, by the USEPA but above the AFSSA recommendations for fish consumption. BSAFs of total PAHs showed differences between target organs, with accumulation in gills and liver found to be double that in muscle. BSAFs of individual compounds were used for inter-species comparison, showing different tendencies between the two species studied. BSAFs have been proposed as a simple screening tool to estimate bioaccumulation in fish, as the first tier of a multi-tier evaluation. Toxic Potency Assessment of sediments for fish show that 60% of sediments are above the Safety Sediment Value formulated for fish chronic exposure situations. Regarding histopathology results, correlations have been found between several alterations and concentrations of PAHs in the abiotic compartments, mainly the water column. The IPAT index of pathologies shows a relatively low impact on fish health. In this work, the use of feral finfish in PAH risk assessment for chronically polluted environments has been proven to be a useful tool for complementing environmental diagnoses and improving their accuracy. This approach combines the measurement of total concentrations in different target organs of several appropriate species, the study of histopathological effects, and correlations between all these results

and the concentrations found in associated sediments and column water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2013.10.062>.

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