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Biological responses and toxicopathic effects elicited in *Solea senegalensis* juveniles by waterborne exposure to benzo[a]pyrene

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are priority contaminants in coastal and estuarine ecosystems under anthropogenic pressure. Although PAHs tend to accumulate in the sediment, toxicity for benthic flat fish such as soles may be caused by PAHs released from the sediment to the water column. Within this context, the present investigation aims at recognizing toxicopathic effects elicited after waterborne exposure to benzo[a]pyrene B[a]P, a model individual PAH compound, in juvenile *Solea senegalensis*. Sole juveniles were exposed to various concentrations of waterborne B[a]P for 3 and 7 days. Brain, liver, gills and gonad were the target tissues selected to determine biochemical and lysosomal biomarkers, and histopathology. Biological responses and toxicopathic effects were consistent with B[a]P concentration and exposure time. From day 3, hepatic catalase inhibition indicated potential oxidative effects of B[a]P. At day 7, contaminant exposure produced hepatic glutathione-*S*-transferase induction at low concentrations and inhibition at higher levels, evidencing a bell-shaped response. A clear gradient in lysosomal membrane destabilisation was observed in relation with B[a]P concentrations. Histopathological lesions were more frequent at day 7 and at higher contaminant levels. It seems that environmentally relevant waterborne concentrations of B[a]P (1000 ng/l) would suffice to cause toxicopathic effects on sole juveniles in relatively short exposure times. In agreement, the Integrative Biological Response index (IBR/n) indicated a dose-dependent decline in health condition upon exposure to B[a]P ($IBR/n_{HighB[a]P} > IBR/n_{MidB[a]P} > IBR/n_{LowB[a]P} > IBR/n_{DMSO} > IBR/n_{Control}$). Overall, changes in antioxidant enzymes activity, lysosomal biomarkers and gill and liver histopathology are responsive early-warning signs of health disturbance in sole juveniles exposed to waterborne PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are present in the aquatic environment and in particular in coastal and estuarine areas where the anthropogenic pressure is significant (industrial discharges, urban runoff, among other sources). PAHs are toxic to marine organisms, in which they can be bioaccumulated or metabolised (Solé et al., 2008; Trisciani et al., 2011; Ruiz et al., 2012; Larcher et al., 2014; Vieweg et al., 2018). Their ubiquitous presence in the environment and toxicity to the marine biota classify them as high priority contaminants that should be surveyed (Directive 2000/60/EC). Due to their hydrophobic nature, PAHs tend to accumulate in the sediment, but they also occur dispersed in water because of bioturbation and biotransformation processes (Tronczynski, 1992; Zhang et al., 2000; Eggleton and Thomas, 2004; Neff et al., 2005; Cousin and Cachot, 2014; Briaudeau et al., 2020). Accordingly, flatfish have attracted great interest in PAHs toxicity assays due to their benthic behaviour that closely relates their health status to both water and sediment contamination. In particular, juvenile soles are used in the field as sentinels for the environmental risk assessment of estuarine ecosystems (Gonçalves et al., 2013; Solé et al., 2013; Briaudeau et al., 2019).

Overall, altered health condition has been reported in sole exposed to PAHs both in the field (Costa et al., 2009a, 2009b; Oliva et al., 2010; Briaudeau et al., 2019) and upon laboratory exposure conditions (Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; Solé et al., 2008; Trisciani et al., 2011). Nevertheless, most laboratory studies assessing

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the toxicopathic effects of PAHs in soles were based on PAHs mixtures whilst exposures to individual model compounds such as benzo[a]pyrene are scarce (Costa et al., 2010; Wessel et al., 2010; Aceña et al., 2017; Aráujo et al., 2019). The use of single model contaminants in laboratory conditions is essential to establish an association in the field between environmental levels of contaminants and alterations in the health status of sentinel species. More short-term toxicity assays using single PAH model contaminants are necessary to understand biological responses elicited in soles upon PAH exposure.

Various alternative combinations of biochemical, cellular and tissue-level biomarkers used as a battery may provide diverse means for assessing the biological effects of PAHs on marine organisms (UNEP/RAMOGE, 1999; Davies and Vethaak, 2012; OSPAR Commission, 2013). In fish, exposure to PAHs may lead to changes in antioxidant enzymes activities such as glutathione-*S*-transferase (GST), catalase (CAT) and superoxide dismutase (SOD), which are considered early signals of oxidative stress in response to exposure to pollutants (Jee and Kang, 2005; Wu et al., 2006; Oliva et al., 2010). Accordingly, these enzyme activities are induced in sole and other fish species exposed to PAHs (Jiménez-Tenorio et al., 2008; Salamanca et al., 2008; Fonseca et al., 2011; Díaz-Garduño et al., 2018). The inhibition of brain acetylcholinesterase (AChE), an enzyme involved in neural transmission, is used as biomarker of neurotoxicity in sole (López-Galindo et al., 2010a, 2010b; Solé et al., 2012; Jebali et al., 2013) and in other fish species (Minier et al., 2000a; Davies and Vethaak, 2012; Burgeot et al., 2017). Lysosomal biomarkers are not pollutant specific but may be indicative of general stress responses in aquatic organisms (UNEP/RAMOGE, 1999; JAMP, 2003; ICES, 2011; 2015; Davies and Vethaak, 2012). Lysosomal enlargement, membrane destabilisation and changes in lysosomal content occur in the hepatocytes of a variety of fish species upon exposure to PAHs and other pollutants (Broeg et al., 2002; Au, 2004; Alvarado et al., 2005; Baršienė et al., 2006; Bilbao et al., 2006; Zorita et al., 2008; Burgeot et al., 2017). Finally, PAHs are known to provoke histopathological lesions in fish gills, liver (Myers et al., 2003; Feist et al., 2004; ICES, 2006; Lang et al., 2006; Salamanca et al., 2008; Costa et al., 2010) and gonads (Minier et al., 2000b; Blazer, 2002; Reynolds et al., 2003; Stentiford and Feist, 2005; Bizarro et al., 2014; Solé et al., 2016).

The present investigation aims at recognizing biological responses and toxicopathic effects elicited after waterborne exposure to B[a]P, a model individual PAH compound, in juvenile *Solea senegalensis*. Biomarkers of oxidative stress and neurotoxicity, lysosomal biomarkers and histopathology were determined after 3 and 7 d exposure and integrated into the Integrative Biological Response (IBR) index (Beliaeff and Burgeot, 2002). Over the last decade, juvenile and adult soles have been used as sentinel organisms for the assessment of marine pollution in European southwestern waters (Oliva et al., 2010, 2012a, 2013; Cuevas et al., 2015; Solé et al., 2016; Briaudeau et al., 2019). Therefore, the outcomes of the present study are relevant to promote the use of juvenile soles as sentinel species for the assessment of biological effects of PAHs in fish from estuarine and coastal ecosystems.

2. Material and methods

2.1. Experimental design

Solea senegalensis juveniles (24.3 ± 2.0 cm length; 160.3 ± 39.6 g total wet-wt) were exposed for 7 d to different nominal concentrations of benzo[a]pyrene (B[a]P) using dimethyl sulfoxide (DMSO) as a carrier (Control; DMSO; Low B[a]P: 100 ng/l; Mid B[a]P: 1000 ng/l and High B[a]P: 100000 ng/l). Stocking density was 4–6 kg/m^2. The Control group did not receive any contaminant or carrier. All other experimental groups received the same concentration of DMSO (0.005% v/v). Each experimental group (n = 26) was placed in a closed-system in a 500 l capacity tank with continuous aeration. Water changes were performed every second day to ensure optimal water conditions: pH = 8, salinity = 31–33 PSU, temperature = 13-14 °C, dissolved O_2 = 6–8 mg/l and total ammonia = 0 mg/l. Photoperiod throughout the experiment was set at 12:12 h light:dark. Fish were daily fed with commercial food (0.3 g per fish; BioMar Iberia S.A., Dueñas, Spain). Biological samples (liver, gills, brain and gonad) were collected from each experimental group after 3 and 7 d of exposure to B[a]P. Liver samples (n = 13) were used for histochemical and histopathological analysis and a section of 6 of these livers were also used for biochemical analysis. Gills and gonad samples (n = 13) were used for histopathological analysis and brain samples (n = 6) were used for biochemical analysis.

2.2. Chemical analysis of water samples

Water samples were collected from each experimental group, 48 h after contaminant load. Analyses of B[a]P content in water were determined by solid-phase microextraction (SPME; Ouyang and Pawliszyn, 2006) and gas chromatography-mass spectrometry analysis (GC-MS).

2.3. Fish biometry

Individual wet-wt (W in g) and length (L in cm) and liver and gonad wet-wt (LW and GW in g, respectively) were recorded to calculate (a) K=W × 100/l^3; (b) HSI = LW × 100/W; and (c) GSI = GW × 100/W; where K is the condition factor, HSI is the hepatosomatic index, and GSI is the gonadosomatic index.

2.4. Biochemical analysis

At days 3 and 7 of exposure, liver and brain samples (n = 6 per experimental group) were dissected out, rapidly frozen and maintained at −80 °C until use. Samples were processed for biochemical analysis; they were homogenised (1:4 for liver and 1:5 for brain) in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged for 30 min at 12,000 *g* at 4 °C to obtain the post-mitochondrial supernatant (PMS). Acetyl cholinesterase (AChE) activity was determined in brain PMS and glutathione-*S*-transferase (GST), catalase (CAT), superoxide dismutase (SOD) enzyme activities in liver PMS, using a BioTek Eon microplate spectrophotometer. Enzyme activities were expressed as a function of the protein concentration in the samples. Total protein content in the homogenates (prot) was measured in triplicate at 595 nm following Bradford's method adapted to microplate and using bovine serum albumin(BSA) as standard (Guilhermino et al., 1996). All enzyme assays were performed at 25 °C.

AChE activity was determined according to the Ellman's colorimetric method adapted to microplate (Guilhermino et al., 1996) at 412 nm. AChE activity was expressed as specific activity (nmol DTNB/min/mg prot). GST activity was determined by the Habig's method adapted to microplate and using bovine serum albumin as standard (Guilhermino et al., 1996). Enzyme activity was measured at 340 nm for 6 min and expressed as nmol/min/mg prot. CAT activity was determined by the method of Claiborne (1985) as absorbance decrease at 240 nm. Results were expressed as μmol H_2O_2/min/mg prot. SOD activity was determined by a colorimetric method using a SIGMA kit (SOD Determination kit; ref: SIGMA, 19160) at 450 nm and SOD activity (inhibition rate %) was calculated following manufacturer's protocol and expressed as nmol/min/mg prot.

2.5. Histochemical analysis

At days 3 and 7 of exposure, liver samples (n = 13 per experimental group) were dissected out, rapidly frozen and maintained at −80 °C until use. Samples were processed for histochemical analysis using the Tissue Array (TA) technology (Array Mold® Kit; n° 20015-A) and TA blocks were cut at-27°C using a Leica CM 3050S cryotome.

Lysosomal membrane stability (LMS) was determined on the basis of the time of acid labilisation treatment required to produce the maximum

staining intensity according to UNEP/RAMOGE (1999), after demonstration of acid phosphatase (AcP) activity in hepatocyte lysosomes. The time of acid labilisation treatment required to produce the maximum staining intensity was assessed under a light microscope and was denoted as the Labilisation Period (LP; in min). Four determinations were made per individual; for each area, the first maximum staining peak was considered to determine the LP value (ICES, 2015). A final LP value was calculated for each individual fish as the mean of the four LP values determined in each area.

Changes in the size and numbers of lysosomes were determined according to the method described by Cajaraville et al. (1989) for mussels, subsequently adapted to fish liver by Alvarado et al. (2005), after histochemical demonstration of β-glucuronidase activity in fish hepatocytes. The structure of lysosomes was assessed through a stereological procedure based on image analysis (BMS, Sevisan) according to Cajaraville et al. (1991). Five measurements using a 100× objective lens were made per individual. The mean value of the following stereological parameters was determined for the lysosomes of each liver sample (Lowe et al., 1981): volume density ($Vv_L = V_L/V_C$), surface density ($Sv_L = S_L/V_C$), surface-to-volume ratio ($S/V_L = S_L/V_L$) and numerical density ($Nv_L = N_L/V_C$); where V = volume, S = surface, N = number, L = lysosomes and C = liver cytoplasm.

Changes in levels of intracellular accumulation of neutral lipids were determined according to Marigómez and Baybay-Villacorta (2003), after Oil Red O (ORO) staining to visualise neutral lipids. Five measurements using a 40× objective lens were made per individual. The mean value of the volume density ($Vv_{NL} = V_{NL}/V_C$) of neutral lipids was determined; where V = volume, NL = neutral lipids and C = liver cytoplasm.

2.6. Histological processing and histopathological examination

At days 3 and 7 of exposure, gill, liver and gonad samples were dissected out (n = 13 per experimental group). Gills were fixed in Bouin's solution for 24hr at 4 °C and rinsed in formic acid (8% v/v) for 24hr at room temperature. Liver and gonad samples were fixed in 4% neutral buffered formaldehyde for 24 h at 4 °C. Fixed samples were dehydrated in a graded series of ethanol, cleared and embedded in paraffin (Leica ASP 300S). A minimum of two sections (5 μm) per sample were obtained using a rotary microtome (Leica RM 2125RTS) and were stained with haematoxylin-eosin (H&E).

Gonad histological sections were analysed at a light microscope for determining gender and gamete developmental stages. Male gamete developmental stages were determined according to García-López et al. (2006) and were classified in five stages as follow: Stage I (early spermatogenesis); Stage II (mid spermatogenesis); Stage III (late spermatogenesis); Stage IV (mature); Stage V (recovery). The identification of gamete developmental stages for females was mainly based on Murua and Motos (2006). Stages were classified as followed: Stage I (growth); Stage II (early vitellogenesis); Stage III (late vitellogenesis); Stage IV (maturation).

The examination of histological samples was made under a light microscope (Nikon Eclipse E200) starting with a 4× objective lens for a general description of the organs followed by higher power objective lenses (10× , 20× , 40× and 100×). For the identification of histopathological lesions on different organs, previous publications were considered (ICES, 1997; BEQUALM, 2001; Blazer, 2002; Costa et al., 2009a; Zorita and Cuevas, 2014).

The prevalence of each histopathological alteration was determined as the percentage occurrence of an alteration within each experimental group for gills and liver and within each combination of experimental group and gender for gonads. Amongst lesions identified, only persistent cases were considered for the calculation of histopathological indices and point alterations were discounted.

The weighted histopathological index developed by Bernet et al. (1999) was applied to liver, gills and gonad. Briefly, each organ lesions were classified into five categories based on their reaction pattern: (1) circulatory disturbances; (2) inflammatory responses; (3) regressive changes; (4) progressive changes; and (5) tumours. Each alteration was assigned an importance factor or weight (w) ranging from 1 to 3: (1) minimal; (2) moderate; and (3) severe. In parallel, the score value (a) of each lesion identified was ranked in four categories (0, 2, 4 and 6) according to the size of the tissue area affected in the sections and the degree of cellular change observed. Finally, semi-quantitative histopathological indices (I) were calculated for each individual and each organ as $I = \sum_{j=1}^{n} w_j \times a_j$; where "$w_j$" is the weight of the j^{th} histopathological alteration and "a_j" the score given to the j^{th} alteration for the individual *h*.

2.7. Integrative Biological Response (IBR/n) index

The IBR index (Beliaeff and Burgeot, 2002; Devin et al., 2014) was calculated based on the integration of biomarkers (GST, CAT, LP) and histopathological indices (I_{gills}, I_{liver}) following the calculation method described by Marigómez et al. (2013) as previously applied in soles (Briaudeau et al., 2020).

2.8. Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics Base 22.0. Normality of data (Shapiro's test) and homogeneity of variance (Levene's test) were tested before statistical analysis. Two-way ANOVA tests were performed to analyse the effects of the B[a]P waterborne concentration ([B[a]P]), the exposure time (T) and their combination ([B[a]P] × T) on biomarkers and histopathology. Logarithmic transformation was applied to non-parametric variables (GST, LP, Vv_L, $S/V_L$and Vv_{NL}). For normal data, differences between experimental groups and throughout exposure time were tested using the parametric one-way ANOVA test and the Student's *t*-test, respectively. For non-normal data set, the non-parametric Kruskal-Wallis' test and Mann-Whitney's *U* test were used to analyse differences in biological data between experimental groups and throughout exposure time. The z-score test and the Pearson's χ^2-test were used to determine significant differences in histopathological lesion prevalence between experimental groups and throughout exposure time. Significant differences in IBR indices from different experimental groups of the same sampling day were tested using the z-score test. Significant differences in chemical data were tested with the z-score test. Level of significance for all analyses was $p = 0.05$.

3. Results

Overall, concentrations of B[a]P measured in water samples 48 h after contaminant loading were lower than the nominal concentrations. Nevertheless, the B[a]P concentration gradient was maintained. Thus, the highest waterborne concentration was measured in the High B[a]P group (17000 ng/l) and the lowest in the Low B[a]P group (40 ng/l), with the Mid B[a]P group (260 ng/l) in between. B[a]P concentrations in the DMSO and Control groups were below detection limits.

In total, 130 individuals were used. Length (24.3 ± 2.0 cm) and W (160.3 ± 39.6 g) were constant throughout the experiment. Likewise, the condition index K (1.1 ± 0.1), HSI (1.0 ± 0.3) and male GSI (0.05 ± 0.02) did not differ between exposure groups. In contrast, female GSI varied between groups at day 7, with the lowest index recorded in Mid B[a]P exposed soles (0.88 ± 0.08) and the highest in DMSO exposed soles (1.08 ± 0.08).

Brain AChE activity was significantly affected by [B[a]P], T and [B[a]P] × T (2-way ANOVA; Table 1), varying from 110.2 ± 2.0 nmol/min/mg prot in Mid B[a]P soles at day 3 to 66.1 ± 3.1 nmol/min/mg prot in Low B[a]P soles at day 7 (Fig. 1A). At day 3, the lowest AChE activity was recorded in Control soles and the highest in DMSO and Mid

Table 1
Summary of the 2-way ANOVAs performed to analyse the effects of $[B[a]P]_{sw}$ (d.f.: 4), time of exposure (d.f.: 1) and their combination ($[B[a]P] \times T$, d.f.: 4) on biomarkers and histopathology (lesion stages and indices) in *S. senegalensis* exposed to concentrations of [B[a]P] for 3 and 7 days. Logarithmic transformation was applied to GST, LP, Vv_L, S/V_L and Vv_{NL} (non-parametric variable). No significant effect of [B[a]P],T or $[B[a]P] \times T$ was detected for Nv_L, and male I_{go} d.f.: degrees of freedom; F: Fisher's F; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Parameter	Residual d.f.	$F([B[a]P]_{sw})$	F(T)	$F([B[a]P]_{sw}xT)$
AChE	50	3.134*	68.314***	9.346***
GST	51	27.579***	0.715	7.275***
CAT	52	16.157***	1.428	1.635
SOD	59	176.035***	1568.478***	23.649***
LP	105	63.576***	22.955***	1.332
Vv_L	95	0.995	38.507***	4.302**
S/V_L	95	4.287**	36.646***	5.013**
Vv_{NL}	90	3.462*	6.630*	2.427
I_{tot}	129	5.345**	1.370	2.477*
I_{gills}	129	4.561**	4.678*	5.322**
I_{liver}	129	3.656**	0.286	2.696*
$FemaleI_{gonad}$	61	6.456***	2.827	0.848

B[a]P treated soles. However, at day 7, AChE activity decreased in DMSO and B[a]P treated soles and reached levels similar to Control values.

Hepatic GST activity was affected by [B[a]P] and $[B[a]P] \times T$ (2-way ANOVA; Table 1). Thus, the lowest GST activity values were recorded in High B[a]P soles at day 3 and in Mid B[a]P and High B[a]P soles at day 7 (Fig. 1B). The highest enzyme activity was measured in Low B[a]P exposed soles at day 7. Hepatic CAT activity was significantly affected by [B[a]P] (2-way ANOVA; Table 1). Thus, the lowest CAT activity was recorded in Mid B[a]P and High B[a]P soles (Fig. 1C). At day 7, soles exposed to Low B[a]P also showed lower enzyme activity than Control and DMSO soles. Enzymatic activities recorded from the Control and DMSO groups were comparable and constant throughout the experiment. Hepatic SOD activity was affected by [B[a]P], T and $[B[a]P] \times T$ (2-way ANOVA; Table 1). For all experimental groups, levels of SOD activity were higher at day 7 than at day 3 and Control soles showed significantly highest enzyme activity at days 3 and 7 (Fig. 1D).

Lysosomal LP was significantly affected by [B[a]P] and T (2-way ANOVA; Table 1); ranging from 30.5 ± 1.6 min in Control soles at day 7 to 5.7 ± 0.4 min in High B[a]P soles at day 3 (Fig. 2A). At day 3 and day 7, LP values from B[a]P exposed soles were significantly lower than levels recorded in the Control groups, in particular for High B[a]P exposed soles. Vv_L and S/V_L were significantly affected by T and $[B[a]P] \times T$ and S/V_L was also affected by [B[a]P] (2-way ANOVA; Table 1). At day 3, Vv_L and S/V_L were similar in all experimental groups whilst at day 7, Vv_L increased and S/V_L decreased in DMSO and B[a]P exposed soles (Fig. 2B and C). Levels of Nv_L (0.00036 ± 0.00002 $1/\mu m^3$) did not vary between experimental groups or exposure days (Fig. 2D). Vv_{NL} was significantly affected by [B[a]P] and T (2-way ANOVA; Table 1). Levels of Vv_{NL} did not vary between experimental groups at day 3 (Fig. 2E). Higher Vv_{NL} values were recorded at day 7 in DMSO and Low B[a]P treated soles.

Most soles examined for histopathology presented lamellar capillary aneurysm and epithelial lifting in the gills (Fig. 3A and B), showing prevalence values ranging from 76.9% to 100% considering all experimental groups (Table 2). Hypertrophy of squamous epithelium was detected at mild to high prevalence (23.1–84.6%) and chloride cell hyperplasia (Fig. 4C) at low to mild prevalence (23.1–69.2%) (Table 2). No tumours were detected in gills.

Often, liver samples appeared largely vacuolated showing important lipid accumulation in hepatocytes in all experimental groups. Haemorrhage, hyperaemia (Fig. 3D) and lymphocytic infiltration were rarely observed in Control and DMSO soles but they were occasionally identified in B[a]P exposed soles (Table 2). Presence of melanomacrophage centres (MMCs; 15.4–76.9%), necrosis (46.2–84.6%; Fig. 3E) and concentric periductal fibrosis in bile ducts (16.7–69.2%; Fig. 3F) were detected at moderate prevalence in most groups (Table 2).

Upon microscopic examination of gonad tissue, most soles were shown to be at an early stage of gamete development. Males mostly presented immature testis (46.3%) and early spermatogenesis stage (50.7%); and although one case of early vitellogenesis (Stage II) was identified, most females (98.4%) presented primary growth oocytes

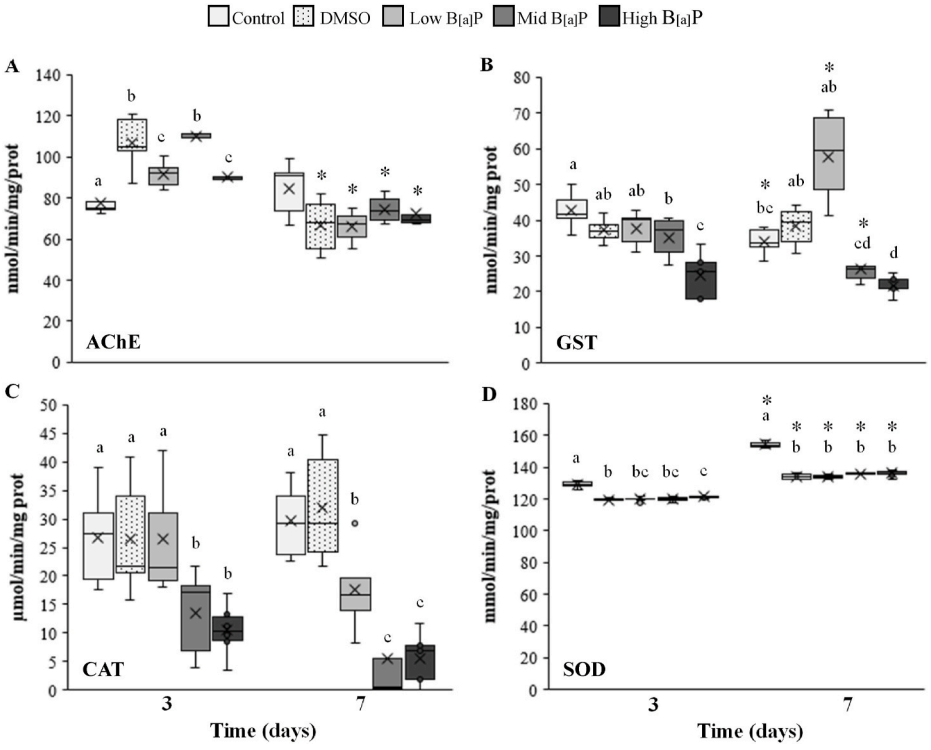

Fig. 1. Brain acetylcholinesterase (A) and hepatic glutathione-*S*-transferase (B), catalase (C) and superoxide dismutase (D) enzyme activities measured in *S. senegalensis* exposed to different concentrations of B[a]P for 3 and 7 d. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p < 0.05$); AChE: acetylcholinesterase; CAT: catalase; GST: glutathione-*S*-transferase; SOD: superoxide dismutase.

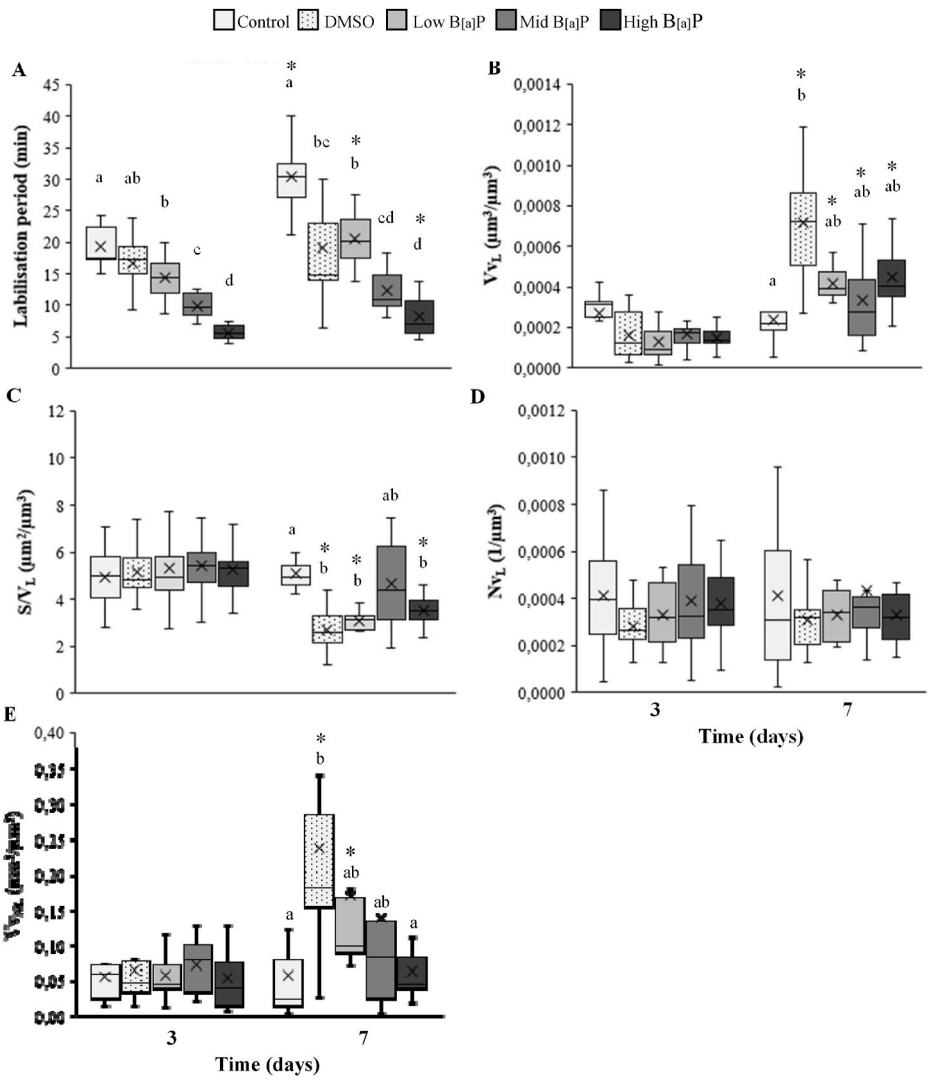

Fig. 2. Lysosomal membrane stability (A), lysosomal structural changes (B–D) and intracellular neutral lipid volume density (E) assessed in liver of *S. senegalensis* exposed to different concentrations of B[a]P for 3 and 7 d. Different letters indicate significant differences between experimental groups of a same sampling time; asterisks indicate significant differences between exposure times ($p < 0.05$). LP: labilisation period; Vv_L: lysosomal volume density; S/V_L: lysosomal surface to volume ratio; Nv_L: lysosomal numerical density; Vv_{NL}: volume density of neutral lipids.

(Stage I). No histopathological lesions were identified in testis of Control soles. Moreover, the only lesions recorded in testis of DMSO and B[a]P exposed soles were scarce with only few cases of granulomatous tissue (<20.0%) and necrosis (<35.0%). In females, lymphocytic infiltration, necrosis, atresia and pre-vitellogenic oocyte lipidosis were rarely detected in Control soles and were at mild to moderate prevalence in the other experimental groups (Table 2).

I_{tot}, I_{gills} and I_{liver} were significantly affected by [B[a]P]and [B[a]P] × T; whereas I_{gills} also varied with T (2-way ANOVA; Table 1). I_{tot}, I_{gills} and I_{liver} from the Control and DMSO groups were similar whilst higher values were recorded in B[a]P exposed soles, in particular at day 7 (Fig. 4A–C). Similarly, female I_{gonad} was significantly affected by B[a]P (2-way ANOVA; Table 1). Although female I_{gonad} did not differ between experimental groups at day 3, highest values were recorded in Mid B[a]P and High B[a]P groups at day 7 (Fig. 4D).

The IBR/n index ranged from 0.01 in Control soles at day 7 to 3.63 in soles exposed to High B[a]P for 7 d (Fig. 5A and B). Higher IBR/n index levels were recorded in High B[a]P soles at days 3 and 7 and in Mid B[a]P soles at day 7.

The successive biological responses elicited by B[a]P were depicted by radar plot profiles: altered antioxidant enzyme activities and lysosomal system anticipated histopathological lesions. At day 3, the main contributors to IBR/n were biochemical responses related to antioxidant defence (GST and CAT) along with altered lysosomal system and to a lesser extent, liver histopathology. Meanwhile, all the selected biological responses contributed to IBR/n at day 7. Whilst the response profile in sole juveniles exposed to 100 ng B[a]P/l was comparable to the one depicted in the DMSO group, nominal B[a]P concentrations in the range of 1000–100000 ng B[a]P/l provoked GST and CAT inhibition, severe lysosomal membrane destabilisation and outstanding histopathological lesions in liver and, most remarkably, in gills.

4. Discussion

The application of individual model PAH compounds like B[a]P for ecotoxicological studies implies the need of a carrier to counteract the hydrophobicity of the contaminant. DMSO has been previously applied as an effective solvent vehicle for organic compounds that is considered to have a low toxicity for fish species (Willford, 1967; Hutchinson et al., 2006). Pawlowski et al. (2004a; b) demonstrated that DMSO at 0.01% (v/v) may affect egg production in fathead minnow but did not alter liver and gonad histology. Similarly, a long-term exposure (100 d) to 0.01–2.0% (v/v) DMSO did not generate histopathological lesions in gills and kidney in Coho salmon (Benville et al., 1968). Thus, the DMSO concentration used herein (0.005% v/v) was not expected to elicit confounding biological responses in sole juveniles. Accordingly, only minor differences were occasionally found between soles of Control (seawater) and DMSO experimental groups. Yet, a 7 d exposure to DMSO seemed to have some influence on SOD enzyme activity and lysosomal biomarkers. Nevertheless, although the toxicity of the carrier used

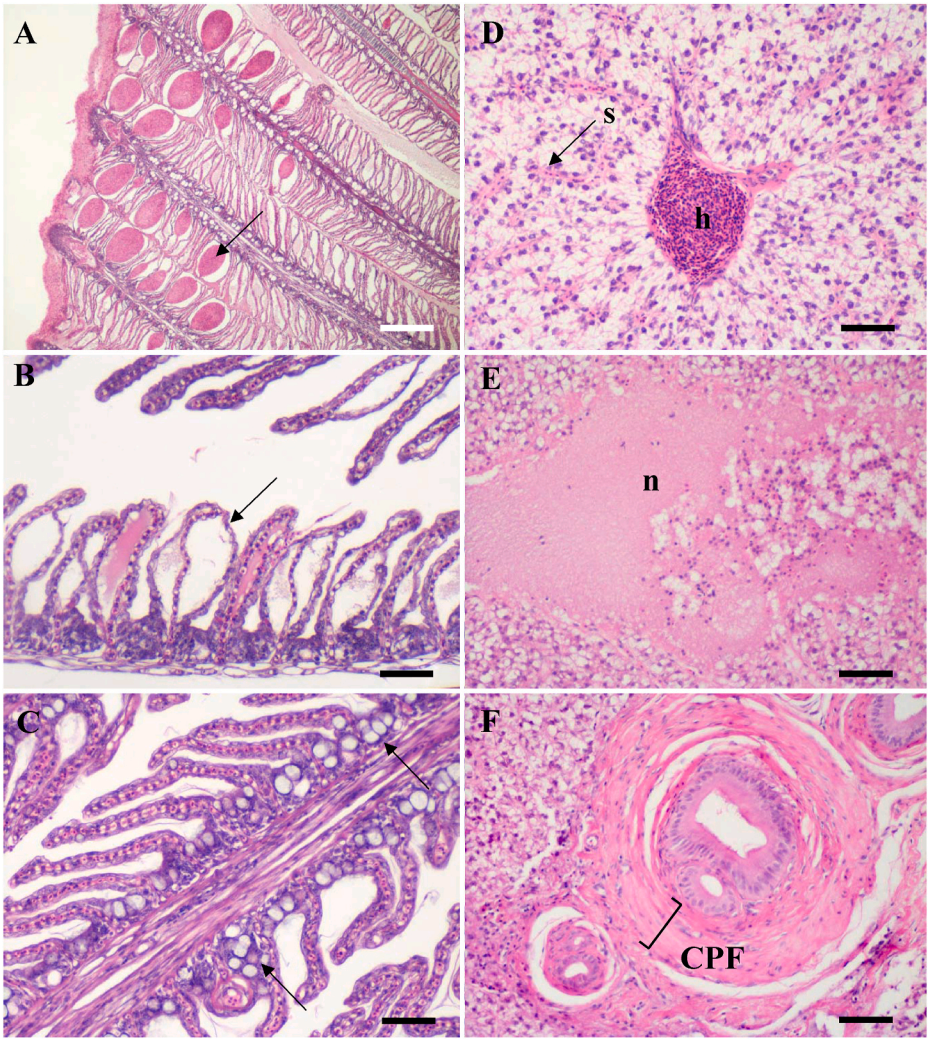

Fig. 3. Histological sections (5 μm) of *S. senegalensis* exposed to different concentrations of B[a]P for 3 and 7 d, stained with haematoxylin and eosin. (A) Gill tissue showing capillary aneurysm (arrow); (B) gill epithelial lifting (arrow); (C) chloride cell hyperplasia; (D) hepatic hyperaemia with accumulation of erythrocytes in blood vessels; h: hyperaemia; s: sinusoid; (E) severe case of hepatic necrosis; (F) Concentric Periductal Fibrosis. Black scale bar: 50 μm; white scale bar: 200 μm.

herein cannot be fully neglected it is worth noting that the responses to B[a]P exposure were clearly demonstrable in comparison with the Control and DMSO groups.

The B[a]P concentrations used in the present study were selected to ensure that short-term biological effects would be exerted in sole juveniles, as it could be expected after preceding reports on other fish species using higher concentrations than used herein (Vieira et al., 2008; Gravato and Guilhermino, 2009). Although only the lowest concentration (100 ng/l) could be considered ecologically realistic, the use of two higher non-realistic concentrations in the present experiment, allowed for the understanding of biological responses in sole juveniles based on the application of a battery of biomarkers assessed at different biological organisation levels. Chemical analysis of water samples permitted to confirm that the B[a]P concentrations in experimental tanks were congruent with the gradient of nominal concentrations originally selected. Yet, the measured concentrations were noticeably lower than the nominal ones. Indeed, heavy PAHs (>3 rings) such as B[a]P occur at low concentration in soluble forms in seawater due to their low seawater solubility and to their strong binding to particulate matters and solid materials (Qingling et al., 2006). In agreement, waterborne PAH concentration in experimental setups is known to be reduced as a result of their adsorption to particulate matter or to the basin surface, as well as due to their photochemical degradation, biotransformation and uptake by fish (Budzinski et al., 2004). Presently, the consequences of these confounding factors in the mid-term were somehow buffered because seawater and B[a]P were renewed every second day, thus maintaining the differences in the B[a]P exposure levels between the experimental groups even though the measured concentrations of the PAH were lower than the nominal ones.

AChE enzyme activity recorded in soles from the seawater Control group was similar to values reported in farmed individuals (Solé et al., 2008). However, a decrease in AChE activity was recorded at day 7 on exposure to DMSO and to different concentrations of B[a]P. AChE enzyme inhibition was reported previously in fish *Pomatoschistus microps* exposed to B[a]P (Vieira et al., 2008) and in *S. senegalensis* exposed to the biocide sodium hypochlorite (López-Galindo et al., 2010a). Overall, exposure to PAHs such as B[a]P is known to inhibit AChE activity in fish brain (Baršienė et al., 2006). To our knowledge, DMSO was not expected to cause neurotoxic effects at the used concentrations (Yen et al., 2011). Nevertheless, behavioural toxicity of the carrier was reported in early life stage of other fish species exposed to higher DMSO concentrations (Chen et al., 2011) and therefore a certain neurotoxicity of this compound cannot be fully disregarded for sole juveniles under the present experimental conditions.

GST activity values recorded herein for Control and DMSO exposed soles resemble those reported for farmed individuals (Solé et al., 2008) and for wild sole from reference sites (Oliva et al., 2012b; Jebali et al., 2013). On this basis, GST was seemingly induced upon exposure to 100

Table 2
Prevalence (%) for histopathological lesions recorded in gills, liver and female gonad in *S. senegalensis* (n = 13 per experimental group; except for gonad1) after 3 and 7d exposure to B[a]P. Bold values indicate significant differences among experimental groups of a same sampling day (Z score; $p < 0.05$). Asterisks indicate significant differences between sampling days (Pearson's χ^2 test, $p < 0.05$). w: lesion importance factor; MMCs: Melanomacrophage Centres; FV: Fat Vacuolation; HV: Hydropic Vacuolation; CPF: Concentric Periductal Fibrosis.

		Day 3					Day 7				
Lesions	*w*	Control	DMSO	Low B[a]P	Mid B[a]P	High B[a]P	Control	DMSO	Low B[a]P	Mid B[a]P	High B[a]P
Gills											
Circulatory disturbances											
Lamellar capillary aneurysm	1	100.0	100.0	100.0	**76.9**	100.0	92.3	**76.9**	84.6	100.0	100.0
Regressive changes											
Epithelial lifting	1	92.3	**75.0**	92.3	100.0	100.0	**76.9**	100.0	92.3	100.0	100.0
Progressive changes											
Hypertrophy of squamous epithelium	1	**53.8**	76.9	76.9	61.5	**84.6**	**23.1**	84.6	53.8	84.6	76.9
Chloride cell hyperplasia	2	46.2	**69.2**	46.2	**38.5**	53.8	23.1	38.5	**69.2**	30.8	15.4*
Liver											
Circulatory disturbances											
Haemorrhage	1	7.7	0.0	7.7	0.0	15.4	0.0	0.0	0.0	7.7	7.7
Hyperaemia	1	23.1	15.4	23.1	7.7	15.4	7.7	0.0	0.0	15.4	23.1
Inflammatory responses											
MMCs	1	15.4	61.5	53.8	61.5	61.5	69.2*	61.5	76.9	61.5	76.9
Lymphocytic infiltration	2	23.1	15.4	30.8	30.8	15.4	0.0	0.0	15.4	53.8	7.7
Regressive changes											
Necrosis	3	46.2	69.2	61.5	76.9	84.6	76.9	61.5	76.9	76.9	61.5
Progressive changes											
FV of hepatocytes	1	100.0	100.0	100.0	92.3	100.0	92.3	100.0	92.3	100.0	100.0
HV of epithelial cells in bile ducts	2	7.7	7.7	0.0	7.7	15.4	0.0	0.0	38.5*	0.0	0.0
CPF of bile ducts	2	23.1	30.8	30.8	16.7	69.2	53.8	38.5	69.2*	53.8	38.5
Gonad (female)[1]		n = 7	n = 6	n = 8	n = 7	n = 5	n = 10	n = 4	n = 6	n = 4	n = 5
Circulatory disturbances											
Hyperaemia	1	0.0	16.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Inflammatory responses											
Lymphocytic infiltration	2	0.0	16.7	0.0	42.9	20.0	0.0	25.0	0.0	50.0	0.0
Regressive changes											
Necrosis	3	0.0	50.0	25.0	57.1	20.0	10.0	75.0	16.7	75.0	60.0
Atresia	3	0.0	16.7	0.0	0.0	0.0	10.0	0.0	0.0	25.0	60.0*
Progressive changes											
Lipids in oocytes	1	28.6	16.7	37.5	42.9	40.0	20.0	0.0	50.0	50.0	40.0

ng B[a]P/l, which suggests that Phase II detoxification processes were activated. Indeed, GST is a Phase II enzyme known to contribute to PAH biotransformation (van der Oost et al., 2003). Alike, GST induction was described in other fish species after exposure to waterborne B[a]P (Vieira et al., 2008; Gravato and Guilhermino, 2009) and in sole juveniles after exposure to oiled sediments (Salamanca et al., 2008). In contrast, under the present experimental conditions exposure to waterborne B[a]P concentrations in the range of1000–100000 ng B[a]P/l caused GST inhibition. Almeida et al. (2012) also reported that GST enzyme activity decreased in juvenile sea bass exposed to waterborne pyrene. In agreement with the present observations, Gravato and Guilhermino (2009) reported that GST enzyme activity was either induced or inhibited in seabass depending on the contaminant concentration: GST activity was induced after exposure to a nominal concentration of 4000 ng B[a]P/l in seawater and inhibited by higher concentrations (8000–16000 ng/l). Thus, the decrease in GST activity observed herein may imply that the capacity to detoxify B[a]P and its metabolites through the pathway of glutathione conjugation is limited in sole juveniles when waterborne concentrations exceed a nominal concentration of 1000 ng B[a]P/l. This concentration is comparable to the range of B[a]P concentration reported in highly contaminated estuarine waters, which in addition occur along in combination with other chemicals (Maskaoui et al., 2002). Induced catalase activity in fish liver has been described upon exposure to contaminants indicating the activation of antioxidant defences (Jee and Kang, 2005; López-Galindo et al., 2010a; b). However, CAT activity can also result inhibited when the overproduction of ROS exceeds the antioxidant capacity of the cell (Roméo et al., 2000; Kalman et al., 2010; Oliva et al., 2012b). Presently, exposure to a nominal concentration of B[a]P in the range of 1000–100000 ng B[a]P/l clearly inhibited CAT activity at day 3, whereas the inhibition was more attenuated and recorded only after 7 days upon exposure to 100 ng B[a]P/l. Therefore, the level of oxidative stress seems to vary with the B[a]P concentration, with a clear and early excess of ROS in sole juveniles exposed to a waterbone nominal concentration of B[a]P higher than 100 ng B[a]P/l (measured 48 h-[B[a]P] = 40 ng B[a]P/l). Likewise, as a parallel evidence of the antioxidant capacity overload at those exposure concentrations, lysosomal responses revealed signs of membrane disruption and potential pathogenesis (see below). Regarding SOD activity, obtained results are not conclusive as changes in this enzyme activity could not be related to B[a]P exposure; quite the contrary, SOD activity was lower in DMSO and B[a]P exposed soles that in Control ones and higher at day 7 than at day 3 in all the experimental groups. Inconsistent results have been reported in other studies and seem to be related to the complexity and timing of the antioxidant defence network. Thus, for instance, SOD induction was reported in fish upon exposure to waterborne B[a]P (2000–20,000 ng B[a]P/l) for 6 d, but SOD was inhibited after 12 d exposure to 20,000 ng B[a]P/l (Wu et al., 2006).

Preceding laboratory experiments revealed that individual PAH compounds like B[a]P can provoke lysosomal enlargement and membrane destabilisation in marine organisms (Marigómez and Baybay-Villacorta, 2003; Marigómez et al., 2005; Zorita et al., 2008). Present LP values recorded in Control and DMSO groups (≈15–30 min) are similar to those previously recorded in healthy fish (Köhler et al., 1992; Köhler and Pluta, 1995; Broeg et al., 1999, 2002; Viarengo et al., 2007; Zorita et al., 2008). Meanwhile, reduced LP values recorded in B[a]P exposed soles range from 14.42 ± 0.98 (100 ng B[a]P/l) to 5.68 ± 0.39 min (100000 ng B[a]P/l), with a clear concentration-dependent profile. These values are similar to those reported in wild *Platichthys flesus* affected by an oil spill event (Baršienė et al., 2006) and in wild *Limanda limanda* collected from the polluted Seine estuary (Burgeot et al., 2017). Altered lysosomal membrane stability is commonly

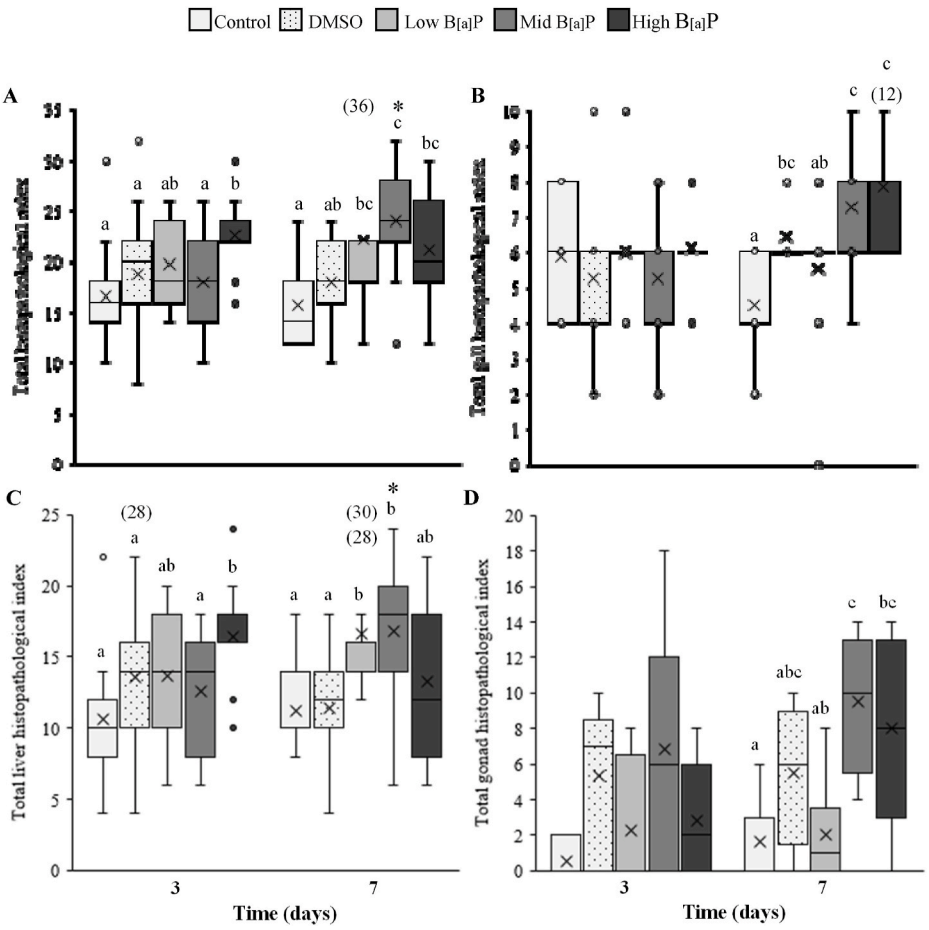

Fig. 4. Total (I_{tot}), gill (I_{gills}), liver (I_{liver}) and female gonad (I_{gonad}) histopathological indices of *S. senegalensis* exposed to different concentrations of B[a]P for 3 and 7 d. Different letters indicate significant differences between experimental groups of the same sampling time; asterisks indicate significant differences between exposure times ($p < 0.05$).

accompanied with lysosomal structural changes evidenced by changes in size and numbers of the lysosomes (Köhler et al., 1992; Cajaraville et al., 1995; Marigómez et al., 2005; Alvarado et al., 2005; Dagnino et al., 2007; Izagirre and Marigómez, 2009). Yet, in the present study lysosomal enlargement (high Vv_L and low S/V_L) was evident only in DMSO and B[a]P exposed soles at day 7, especially in the former. A comparable profile was observed regarding intracellular accumulation of neutral lipids. It seems therefore that the carrier exerted some effects on juvenile soles, thus rendering present results on changes in lysosomal structure and content not fully conclusive regarding B[a]P effects.

Gill lesions identified in the present study were similar to those previously described in wild fish from contaminated sites (Stentiford et al., 2003; Camargo and Martinez, 2007; Oliva et al., 2013; Santos et al., 2014; Briaudeau et al., 2019) and from laboratory experiments using organic compounds (Jímenez-Tenorio et al., 2008; Martins et al., 2016). Overall, higher lesion prevalence were recorded in B[a]P exposed soles than in Control or DMSO groups at day 7. Similarly, higher lesion stage of epithelial lifting was recorded at day 7. Accordingly, the gill histopathological index increased in agreement with B[a]P concentrations. Haemorrhage, hyperaemia, increased MMCs, lymphocytic infiltration, hydropic vacuolation of epithelial cells of bile ducts, concentric periductal fibrosis and necrosis were observed at higher prevalence in B[a]P exposed soles than in Control or DMSO groups. These lesions were previously described in sole, both after laboratory exposure to pollutants and in field studies (Costa et al., 2011, 2013; Oliva et al., 2013; Zorita and Cuevas, 2014; Briaudeau et al., 2019, 2020). In testis, only few cases of granulomatous tissue and necrosis were recorded, with no clear relation with B[a]P exposure. In contrast, lymphocytic infiltration, necrosis, atresia and lipids in oocytes were identified at mild to moderate prevalence in the ovaries of B[a]P exposed soles, especially upon exposure to 1000–100000 ng B[a]P/l. These lesions were previously reported in fish subject to environmental stress (Blazer, 2002; Reynolds et al., 2003; Cuevas et al., 2015a, 2015b; Briaudeau et al., 2019, 2020). Lesions were integrated into weighted histopathological indices (Bernet et al., 1999; Van Dyk et al., 2007; Costa et al., 2009a) to identify potential cause-effect relationship between exposure to B[a]P and disease condition in sole juveniles. Thus, higher total index (I_{tot}), gill index (I_{gills}) and liver index (I_{liver}) were recorded in soles exposed to B[a]P than in the Control and DMSO groups, in particular at day 7. In agreement, previous works reported increased histopathological indices after exposure to pollutants (Bernet et al., 2004; Van Dyk et al., 2007; Jímenez-Tenorio et al., 2008; Costa et al., 2009a; Briaudeau et al., 2019). Moreover, the highest I_{liver} (≈16) and I_{gonad} (≈10) values recorded upon exposure to 1000–100000 ng B[a]P/l are not dissimilar from the values reported in sole juveniles exposed to contaminated sediments (Briaudeau et al., 2020).

The IBR/n index had been successfully applied in laboratory experiments with sole (Briaudeau et al., 2020) and in biomonitoring programmes using diverse sentinel species (Broeg and Lehtonen 2006; Brooks et al., 2011; Serafim et al., 2012; Marigómez et al., 2013; Rementeria et al., 2016). The successive biological responses elicited by B[a]P were depicted by radar plot profiles: altered antioxidant enzyme activities and lysosomal system anticipated histopathological lesions. At day 3, the main contributors to IBR/n were biochemical responses related to antioxidant defense (GST and CAT) along with altered lysosomal system and to a lesser extent, liver histopathology. Meanwhile, all

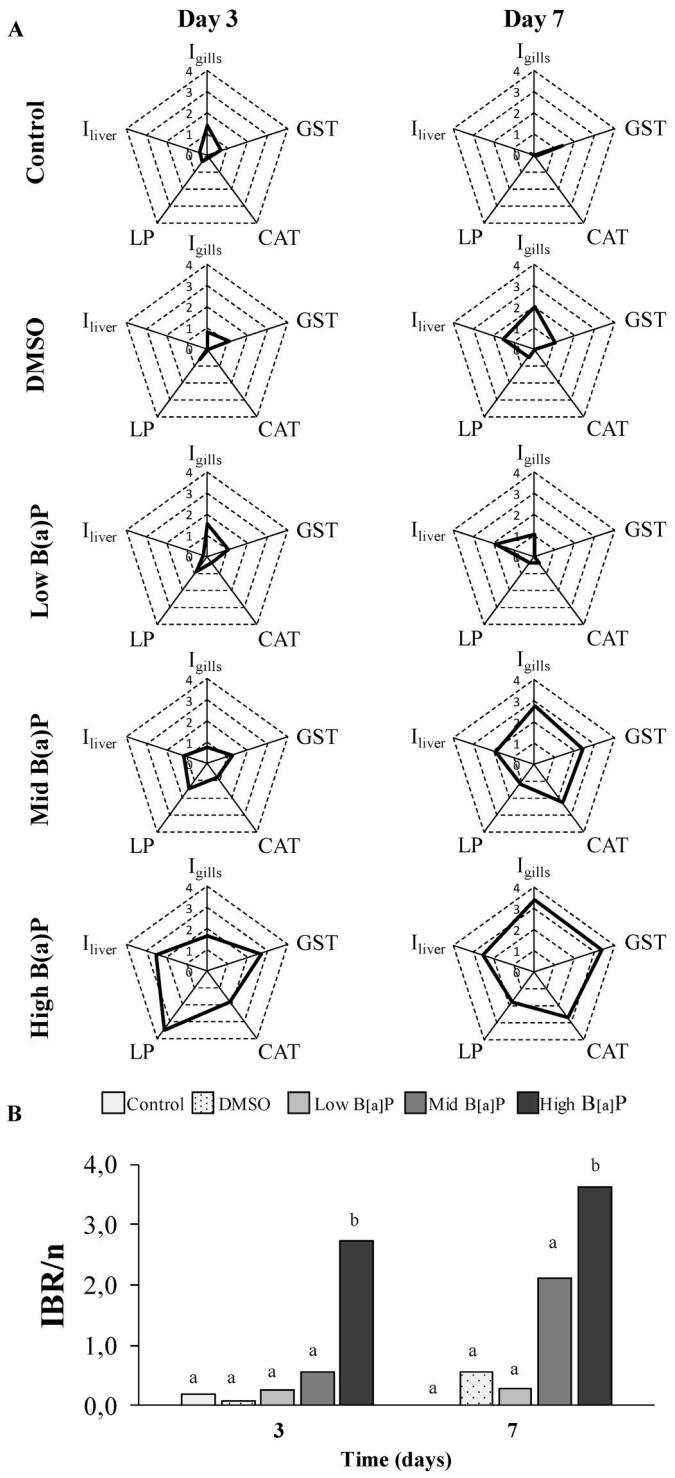

Fig. 5. (A) Radar plots constructed using five selected biological parameters (GST, CAT, LP, I_{liver} and I_{gills}) for each experimental group of juvenile *S. senegalensis* exposed to different concentrations of B[a]P for 3 and 7 d. (B) IBR/n index calculated on the basis of the radar plots.

the selected biological responses contributed to IBR/n at day 7. Whilst the response profile in sole juveniles exposed to 100 ng B[a]P/l was comparable to the one depicted in the DMSO group, exposure to nominal concentrations of B[a]P in the range of 1000–100000 ng B[a]P/l provoked inhibition of GST and CAT enzyme activities, severe lysosomal membrane destabilisation and outstanding histopathological lesions in liver and, most remarkably, in gills. In the present study, IBR/n index was also useful to show, in a user-friendly manner, a dose and time dependent decline in the health condition in sole juveniles exposed to waterborne B[a]P; with a clear gradient in the IBR/n index ($IBR/n_{HighB[a]P} > IBR/n_{MidB[a]P} > IBR/n_{LowB[a]P} > IBR/n_{DMSO} > IBR/n_{Control}$), especially at day 7.

5. Conclusions

The assessment and integration of biological responses elicited in *Solea senegalensis* juveniles upon B[a]P exposure for 7 d indicated different degrees of B[a]P toxicity depending on the waterborne B[a]P concentration and the exposure time. Whilst a 3-d exposure to a nominal concentration in the range of 1000–100,000 ng B[a]P/l caused oxidative stress (CAT inhibition; GST induction/inhibition) and lysosomal membrane destabilisation, a 7-d exposure also caused gill, liver and gonad histopathological lesions. Upon exposure to 100 ng B[a]P/l, the effects were not distinguishable from those elicited by the experimental carrier DMSO. However, they were elicited both upon exposure to a non-environmentally relevant B[a]P concentration (e.g. 100000 ng B[a]P/l) and also upon exposure to an environmentally relevant concentration (1000 ng B[a]P/l). Therefore, it seems that environmentally relevant waterborne concentrations of B[a]P would suffice to cause toxicopathic effects on sole juveniles in relatively short exposure times. Moreover, if B[a]P alone is a potential toxicopathic pollutant, its co-occurrence in the water column with other PAHs and chemicals derived from polluted sediments (Briaudeau et al., 2019, 2020) would be expected to represent a veiled but real hazard that should entail future research efforts.

Author contribution

Tifanie Briaudeau: Methodology, Investigation, Visualization, Writing original draft, Luis Alejandro Alves Dos Santos: Investigation. Izaskun Zorita: Methodology, Formal analysis. Urtzi Izagirre: Conceptualization, Investigation, Writing original draft, Supervision. Ionan Marigómez: Conceptualization, Funding acquisition, Formal analysis, Supervision. Writing, review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Aceña, J., Pérez, S., Eichhorn, P., Solé, M., Barceló, D., 2017. Metabolite profiling of carbamazepine and ibuprofen in *Solea senegalensis* bile using high-resolution mass spectrometry. Anal. Bioanal. Chem. 409, 5441–5450.

Almeida, J., Gravato, C., Guilhermino, L., 2012. Challenges in assessing the toxic effects of polycyclic aromatic hydrocarbons to marine organisms: a case study on the acute toxicity of pyrene to the European seabass (*Dicentrarchus labrax* L.). Chemosphere 86, 926–937.

Alvarado, N.E., Buxens, A., Mazón, L.I., Marigómez, I., Soto, M., 2005. Cellular biomarkers of exposure and biological effect in hepatocytes of turbot (*Scophthalmus*

maximus) exposed to Cd, Cu and Zn and after depuration. Aquat. Toxicol. 74, 110–125.
Araújo, M.J., Quintaneiro, C., Soares, A.M.V.M., Monteiro, M.S., 2019. Effects of triclosan on early development of *Solea senegalensis*: from biochemical to individual level. Chemosphere 235, 885–899.
Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar. Pollut. Bull. 48, 817–834.
Baršienė, J., Lethonen, K., Koehler, A., Broeg, K., VuorinenP, J., Lang, T., Pempkowiak, J., Syvokiene, J., Dedonyte, V., Rybakovas, A., Repecka, R., Vuontisjarvi, H., Kopecka, J., 2006. Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipeda-Buyinge area (Baltic Sea). Mar. Pollut. Bull. 53, 422–436.
Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological. Environ. Toxicol. Chem. 21, 1316–1322.
BenvilleP, E.J., Smith, C.E., Shanks, W.E., 1968. Some toxic effects of dimethyl sulfoxide in salmon and trout. Toxicol. Appl. Pharmacol. 12, 156–178.
BEQUALM, 2001. Biological effects quality assurance in monitoring programmes. http://www.bequalm.org/about.htm.
Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: a proposal for a protocol to assess aquatic pollution. J. Fish. Dis. 22, 25–35.
Bernet, D., Schmidt-Posthaus, H., Wahli, T., Burkhardt-Holm, P., 2004. Evaluation of two monitoring approaches to assess effects of wastewater disposal on histological alterations in fish. Hydrobiologia 524, 53–66.
Bilbao, E., Raingeard, D., Diaz de Cerio, O., Ortiz-Zarragoitia, M., Ruiz, P., Izagirre, U., Orbea, A., Marigómez, I., Cajaraville, M.P., Cancio, I., 2006. 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. 98, 282–296.
Bizarro, C., Ros, O., Vallejo, A., Prieto, A., Etxebarria, N., Cajaraville, M.P., Ortiz-Zarragoitia, M., 2014. Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay). Mar. Environ. Res. 96, 19–28.
Blazer, V.S., 2002. Histopathological assessment of gonadal tissue in wild fishes. Fish Physiol. Biochem. 26, 85–101.
Briaudeau, T., Zorita, I., Cuevas, N., Franco, J., Marigómez, I., Izagirre, U., 2019. Multi-annual survey of health status disturbance in the Bilbao estuary (Bay of Biscay) based on sediment chemistry and juvenile sole (*Solea* spp.) histopathology. Mar. Pollut. Bull. 145, 126–137.
Briaudeau, T., Zorita, I., Izagirre, U., Marigómez, I., 2020. Biological responses and toxicopathic effects elicited in *Solea senegalensis* juveniles on exposure to contaminated sediments under laboratory conditions. Sci. Total Environ. 731, 138849.
Broeg, K., Zander, S., Diamant, A., Körting, W., Krüner, G., Paperna, I., Westernhagen, H. V., 1999. The use of fish metabolic, pathological and parasitological indices in pollution monitoring I. North Sea. Helgol. Mar. Res. 53, 171–194.
Broeg, K., Köhler, A., Westernhagen, H.V., 2002. Disorder and recovery of environmental health monitored by means of lysosomal stability in liver of European flounder (*Platichthys flesus* L.). Mar. Environ. Res. 54, 569–573.
Broeg, K., Lehtonen, K.K., 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. Mar. Pollut. Bull. 53, 508–522.
Budzinski, H., Mazéas, O., Tronczynski, J., Désaunay, Y., Bocquené, G., Claireaux, G., 2004. Link between exposure of fish (*Solea solea*) to PAHs and metabolites: application to the *"Erika"*oil spill. Aquat. Living Resour. 17, 329–334.
Burgeot, T., Akcha, F., Ménard, D., Robinson, C., Loizeau, V., Brach-Papa, C., Martínez-Gómez, C., Le Goff, J., Budzinski, H., Le Menach, K., Cachot, J., Minier, C., Broeg, K., Hylland, K., 2017. Integrated monitoring of chemicals and their effects on four sentinel species, *Limanda limanda, Platichthys flesus, Nucella lapillus* and *Mytilus* sp., in Seine Bay: a key step towards applying biological effects to monitoring. Mar. Environ. Res. 124, 92–105.
Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1989. A stereological survey of lysosomal structure alterations in *Littorinalittorea* exposed to 1-naphthol. Comparat. Biochem. Physiol. 93C, 231–237.
Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1991. Automated measurement of lysosomal structure alterations in oocytes of mussels exposed to petroleum derived hydrocarbons. Arch. Environ. Contam. Toxicol. 21, 395–400.
Cajaraville, M.P., Abascal, I., Etxeberria, M., Marigómez, I., 1995. Lysosomes as cellular markers of environmental pollution: time- and dose dependent responses of the digestive lysosomal system of mussels after petroleum hydrocarbon exposure. Environ. Toxicol. Water Qual. 10, 1–8.
Camargo, M.M.P., Martinez, C.B.R., 2007. Histopathology of gills, kidney and liver of a neotropical fish caged in an urban stream. Neotrop. Ichthyol. 5, 327–336.
Chen, T.-H., Wang, Y.-H., Wu, Y.-H., 2011. Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat. Toxicol. 102, 162–166.
Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods in Oxygen Radical Research. CRC Press, Boca Raton, Florida, pp. 283–284.
Costa, P.M., Caeiro, S., Costa, M.H., 2013. Multi-organ histological observations on juvenile Senegalese soles exposed to low concentrations of waterborne cadmium. Fish Physiol. Biochem. 39, 143–158.
Costa, P.M., Caeiro, S., Diniz, M.S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.A., Costa, M.H., 2009b. Biochemical endpoints on juvenile *Solea senegalensis* exposed to estuarine sediments: the effect of contaminant mixtures on metallothionein and CYP1A induction. Ecotoxicology 18, 988–1000.
Costa, P.M., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelVallsT, A., Costa, M.H., 2011. Estuarine ecological risk based on hepatic histopathological indices from laboratory and in situ tested fish. Mar. Pollut. Bull. 62, 55–65.
Costa, P.M., Chicano-Gálvez, E., López Barea, J., DelValls, T.A., Costa, M.H., 2010. Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmium and benzo[a]pyrene. Environ. Pollut. 158, 3338–3346.
Costa, P.M., Diniz, M.S., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.A., Costa, M.H., 2009a. Histological biomarkers in liver and gills of juvenile *Solea senegalensis* exposed to contaminated estuarine sediments: a weighted indices approach. Aquat. Toxicol. 92, 202–212.
Cousin, X., Cachot, J., 2014. PAHs and fish—exposure monitoring and adverse effects—from molecular to individual level. Environ. Sci. Pollut. Control Ser. 21, 13685–13688.
Cuevas, N., Zorita, I., Costa, P.M., Larreta, J., Franco, J., 2015a. Histopathological baseline levels and confounding factors in common sole (*Solea solea*) for marine environmental risk assessment. Mar. Environ. Res. 110, 162–173.
Cuevas, N., Zorita, I., Costa, P.M., Quincoces, I., Larreta, J., Franco, J., 2015b. Histopathological indices in sole (*Solea solea*) and hake (*Merluccius merluccius*) for implementation of the European marine strategy framework directive along the Basque continental shelf (SE Bay of Biscay). Mar. Pollut. Bull. 94, 185–198.
Dagnino, A., Allen, J.I., Moore, M.N., Broeg, K., Canesi, L., Viarengo, A., 2007. Development of an expert system for the integration of biomarker responses in mussels into an animal health index. Biomarkers 12, 155–172.
Davies, I.M., Vethaak, A.D., 2012. Integrated Marine Environmental Monitoring of Chemicals and Their Effects. ICES Cooperative Research Report No. 315, p. 277.
Devin, S., Burgeot, T., Giambérini, L., Minguez, L., Pain-Devin, S., 2014. The integrated biomarker response revisited: optimization to avoid misuse. Environ. Sci. Pollut. Control Ser. 21, 2448–2454.
Díaz-Garduño, B., Perales, J.A., Biel-Maeso, M., Pintado-Herrera, M.G., Lara-Martin, P. A., Garrido-Pérez, C., Martín-Díaz, M.L., 2018. Biochemical responses of *Solea senegalensis* after continuous flow exposure to urban effluents. Sci. Total Environ. 615, 486–497.
Directive 2000/60/EC, 2000. Directive 2000/60/EC of the European parliament and of the council of 23 october 2000 establishing a framework for community action in the field of water policy. Q. J. Electron. Commer. QJEC 327, 1–72.
Eggleton, J., Thomas, K.V., 2004. A review of factors affecting the release and bioavailability of contaminants during sediment disturbance events. Environ. Int. 30, 973–980.
Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A., 2004. Biological effects of contaminants: use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. In: ICES Techniques in Marine Environmental Sciences No. 38. ICES, Copenhagen.
Fonseca, V.F., França, S., Serafim, A., Company, R., Lopez, B., Bebianno, M.J., Cabral, H. N., 2011. Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentratus labrax, Solea senegalensis* and *Pomatoschistus microps*. Aquat. Toxicol. 102, 216–227.
García-López, A., Fernández-Pasquier, V., Couto, E., CanarioA, V.M., Sarasquete, C., Martínez-Rodríguez, G., 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen. Comp. Endocrinol. 147, 343–351.
Gonçalves, C., Martins, M., Costa, M.H., Caeiro, S., Costa, P.M., 2013. Ecological risk assessment of impacted estuarine áreas: integrating histological and biochemical endpoints in wild Senegalese sole. Ecotoxicol. Environ. Saf. 95, 202–211.
Gravato, C., Guilhermino, L., 2009. Effects of benzo(a)pyrene on seabass (*Dicentrarchus labrax*L.): biomarkers, growth and behavior. Human Ecol. Risk Assess. 15, 121–137.
Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. Bull. Environ. Contam. Toxicol. 57, 979–985.
Hutchinson, T.H., Shillabeer, N., Winter, M.J., Pickford, D.B., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. Aquat. Toxicol. 76, 69–92.
ICES, 1997. Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants. ICES, Copenhagen, p. 75.
ICES, 2006. Report of the ICES/BSRP Sea-Going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM). ICES, p. 89.
ICES, 2011. ICES/OSPAR SGIMC Report 2011. Report of the study group on integrated monitoring of contaminants and biological effects (SGIMC), ICES CM 2011/ACOM: 30.
ICES, 2015. Lysosomal membrane stability in mussels. In: Martínez-Gómez, C., Bignell, J., Lowe, D. (Eds.), ICES Techniques in Marine Environmental Sciences No. 56, p. 41.
Izagirre, U., Marigómez, I., 2009. Lysosomal enlargement and lysosomal membrane destabilisation in mussel digestive cells measured by an integrative index. Environ. Pollut. 157, 1544–1553.
JAMP (Joint Assessment and Monitoring Program), 2003. JAMP Guidelines for Contaminant-specific Biological Effects Monitoring (Oslo and Paris Commissions).
Jebali, J., Sabbagh, M., Banni, M., Kamel, N., Ben-Kheder, S., M'hmadi, N., Boussetta, H., 2013. Multiple biomarkers of pollution effects in *Solea solea* fish on the Tunisia coastline. Environ. Sci. Pollut. Control Ser. 20, 3812–3821.
JeeJ, H., Kang, J.C., 2005. Biochemical changes of enzymatic defence system after phenanthrene exposure in olive flounder, *Paralichthys olivaceus*. Physiol. Res. 54, 585–591.
Jiménez-Tenorio, N., Salamanca, M.J., Garcia-Luque, E., Gonzalez de Canales, M.L., DelValls, T.A., 2008. Chronic bioassay in benthic fish for the assessment of the

quality of sediments in different areas of the coast of Spain impacted by acute and chronic oil spill. Environ. Toxicol. 23, 634–642.
Kalman, J., Riba, I., DelValls, T.A., Blasco, J., 2010. Comparative toxicity of cadmium in the commercial fish species *Sparus aurata* and *Solea senegalensis*. Ecotoxicol. Environ. Saf. 73, 306–311.
Köhler, A., Deisemann, H., Lauritzen, B., 1992. Histological and cytochemical indices of toxic injury in the liver of dab *Limanda limanda*. Mar. Ecol. Prog. Ser. 91, 141–153.
Köhler, A., Pluta, H.J., 1995. Lysosomal Injury and MFO Activity in the liver of flounder (*Platichthysflesus* L.) in relation to histopathology of hepatic degeneration and carcinogenesis. Mar. Environ. Res. 39, 255–260.
Lang, T., Wosniok, W., Baršienė, J., Broeg, K., Kopecka, J., Parkkonen, J., 2006. Liver histopathology in Baltic flounder (*Platichthys flesus*) as an indicator of biological effects of contaminants. Mar. Pollut. Bull. 53, 488–496.
Larcher, T., Perrichon, P., Vignet, C., Ledevin, M., Le Menach, K., Lyphout, L., Landi, L., Clerandeau, C., Lebihanic, F., Menard, D., Burgeot, T., Budzinski, H., Akcha, F., Cachot, J., Cousin, X., 2014. Chronic dietary exposure of zebrafish to PAH mixtures results in carcinogenic but not genotoxic effects. Environ. Sci. Pollut. Control Ser. 21, 13833–13849.
López-Galindo, C., Vargas-Chacoff, L., Nebot, E., Casanueva, J.F., Rubio, D., Solé, M., Mancer, J.M., 2010a. Biomarker responses in *Solea senegalensis* exposed to sodium hypochlorite used as antifouling. Chemosphere 78, 885–893.
López-Galindo, C., Vargas-Chacoff, L., Nebot, E., Casanueva, J.F., Rubio, D., Solé, M., Mancer, J.M., 2010b. Sublethal effects of the organic antifoulant Mexel®432 on osmoregulation and xenobiotic detoxification in the flatfish *Solea senegalensis*. Chemosphere 79, 78–85.
Lowe, D.M., Moore, M.N., Clarke, K.R., 1981. Effects of oil on digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. Aquat. Toxicol. 1, 213–226.
Marigómez, I., Baybay-Villacorta, L., 2003. Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquat. Toxicol. 64, 235–257.
Marigómez, I., Izagirre, U., Lekube, X., 2005. Lysosomal enlargement in digestive cells of mussels exposed to cadmium, benzo[a]pyrene and their combination. Comparat. Biochem. Physiol. 141C, 188–193.
Marigómez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., Cajaraville, M.P., 2013. Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill "Mussel Watch". Ecotoxicology 22, 486–505.
Martins, M., Santos, J.M., Costa, M.H., Costa, P.M., 2016. Applying quantitative and semi-quantitative histopathology to address the interaction between sediment-bound polycyclic aromatic hydrocarbons in fish gills. Ecotoxicol. Environ. Saf. 131, 164–171.
Maskaoui, K., Zhou, J.L., Hong, H.S., Zhang, Z.L., 2002. Contamination by polycyclic aromatic hydrocarbons in the Jiulong river estuary and Western Xiamen sea, China. Environ. Pollut. 118, 109–122.
Minier, C., Levy, F., Rabel, D., Bocquené, G., Godefroy, D., Burgeot, T., Leboulenger, F., 2000a. Flounder health status in the Seine Bay. A multibiomarker study. Mar. Environ. Res. 50, 373–377.
Minier, C., Caltot, G., Leboulenger, F., Hill, E.M., 2000b. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. Endocr. Disruptors 28, 801–806.
Murua, H., Motos, L., 2006. Reproductive strategy and spawning activity of the European hake *Merluccius merluccius* (L.) in the Bay of Biscay. J. Fish. Biol. 69, 1288–1303.
Myers, M.S., Johnson, L.L., Collier, T.K., 2003. Establishing the causal relationship between Polycyclic Aromatic Hydrocarbon (PAH) exposure and hepatic neoplasms and neoplasia-related liver lesions in English Sole (*Pleuronectes vetulus*). Human Ecol. Risk Assess. 9, 67–94.
Neff, J.M., Stout, S.A., Gunster, D.G., 2005. Ecological Risk Assessment of Polycyclic Aromatic Hydrocarbons in Sediments: Identifying Sources and Ecological Hazard. Integrated Environmental Assessment and Management.
Oliva, M., González de Canales, M.L., Gravato, C., Guilhermino, L., Perales, J.A., 2010. Biochemical effects and polycyclic aromatic hydrocarbons (PAHs) in Senegal sole (*Solea senegalensis*) from Huelva estuary (SW Spain). Ecotoxicol. Environ. Saf. 73, 1842–1851.
Oliva, M., Perales, J.A., Gravato, C., Guilhermino, L., Galindo-Riano, M.D., 2012a. Biomarkers responses in muscle of Senegal sole (*Solea senegalensis*) from a heavy metals and PAHs polluted estuary. Mar. Pollut. Bull. 64, 2097–2108.
Oliva, M., Vicente, J.J., Gravato, C., Guilhermino, L., Galindo-Riano, M.D., 2012b. Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): seasonal and spatial variation. Ecotoxicol. Environ. Saf. 75, 151–162.
Oliva, M., Vicente-Martorell, J.J., Galindo-Riaño, M.D., Perales, J.A., 2013. Histopathological alterations in Senegal sole, *Solea senegalensis*, from a polluted Huelva estuary (SW, Spain). Fish Physiol. Biochem. 39, 523–545.
OSPAR Commission, 2013. Background Document and Technical Annexes for Biological Effects Monitoring, Update 2013. OSPAR Commission, London, UK.
Ouyang, G., Pawliszyn, J., 2006. SPME in environmental analysis. Anal. Bioanal. Chem. 386, 1059–1073.
Pawlowski, S., van Aerle, R., Tyler, C.R., Braunbeck, T., 2004a. Effects of 17α-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57, 330–345.
Pawlowski, S., Sauer, A., Shears, J.A., Tyler, C.R., Braunbeck, T., 2004b. Androgenic and estrogenic effects of the synthetic androgen 17α-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. Aquat. Toxicol. 68, 277–291.
QinglingL, I., XiaoqinX, U., Sen-Chun, L.F., Xiaoru, W., 2006. Determination of trace PAHs in seawater and sediment pore-water by solid-phase microextraction (SPME) coupled with GC/MS. Sci. China, Ser. B: Chemistry 49, 481–491.
Rementeria, A., Mikolaczyk, M., Lanceleur, L., Blanc, G., Soto, M., Schäfer, J., Zaldibar, B., 2016. Assessment of the effects of Cu and Ag in oysters *Crassostrea gigas* (Thunberg, 1793) using a battery of cell and tissue level biomarkers. Mar. Environ. Res. 122, 11–22.
Reynolds, W.J., Feist, S.W., Jones, G.J., Lyons, B.P., Sheahan, D.A., Stentiford, G.D., 2003. Comparison of biomarker and pathological responses in flounder (*Platichthys flesus* L.) induced by ingested polycyclic aromatic hydrocarbon (PAH) contamination. Chemosphere 52, 1135–1145.
Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., Girard, J.P., 2000. Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. Aquat. Toxicol. 48, 185–194.
Ruiz, P., Ortiz-Zarragoitia, M., Orbea, A., Theron, M., Le Floch, S., Cajaraville, M.P., 2012. Responses of conventional and molecular biomarkers in turbot *Scophthalmus maximus* exposed to heavy fuel oil no 6 and styrene. Aquat. Toxicol. 117, 116–128.
Salamanca, M.J., Jiménez-Tenorio, N., González de Canales, M.L., DelVallsT, A., 2008. Evaluation of the toxicity of an oil spill conducted through bioassays using the fish *Solea senegalensis*. Cienc. Mar. 34, 339–348.
Santos, D.M.S., Melo, M.R.S., Mendes, D.C.S., Rocha, I.K.B.S., Silva, J.P.L., Cantanhêde, S.M., Meletti, P.C., 2014. Histological changes in gills of two fish species as indicators of water quality in Jansen Lagoon (São Luís, Maranhão State, Brazil). Int. J. Environ. Res. Publ. Health 11, 12927–12937.
Serafim, A., Company, R., Lopes, B., Fonseca, V.F., Franca, S., Vasconcelos, R.P., Bebianno, M.J., Cabral, H.N., 2012. Application of an integrated biomarker response index (IBR) to assess temporal variation of environmental quality in two Portuguese aquatic systems. Ecol. Indicat. 19, 215–225.
Solé, M., Lima, D., Reis-Henriques, M.A., Santos, M.M., 2008. Stress biomarkers in juvenile Senegal sole, *Solea senegalensis*, exposed to the water-accommodated fraction of the "Prestige" fuel oil. Bull. Environ. Contam. Toxicol. 80, 19–23.
Solé, M., Vega, S., Varó, I., 2012. Characterization of type "B" esterases and hepatic CYP450 isoenzymes in Senegalese sole for their further application in monitoring studies. Ecotoxicol. Environ. Saf. 78, 72–79.
Solé, M., Manzanera, M., Bartolomé, A., Tort, Ll., Caixach, J., 2013. Persistent organic pollutants (POPs) in sediments from fishing grounds in the NW Mediterranean: ecotoxicological implications for the benthic fish *Solea* sp. Mar. Pollut. Bull. 67, 158–165.
Solé, M., Mañanós, E., Blázquez, M., 2016. Vitellogenin, sex steroid levels and gonadal biomarkers in wild *Solea solea* and *Solea senegalensis* from NW Mediterranean fishing grounds. Mar. Environ. Res. 117, 63–74.
Stentiford, G.D., Longshaw, M., Lyons, B.P., Jones, G., Green, M., Feist, S.W., 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Mar. Environ. Res. 55, 137–159.
Stentiford, G.D., Feist, S.W., 2005. First reported cases of intersex (ovotestis) in the flatfish species dab, *Limanda limanda*: Dogger Bank, North Sea. Mar. Ecol. Prog. Ser. 301, 307–310.
Trisciani, A., Corsi, I., Della Torre, C., Perra, G., Focardi, S., 2011. 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. 62, 806–814.
Tronczynski, J., 1992. Interactions between dissolved organic matter and organic contaminants. Anal. Mag. 20, 54–56.
UNEP/RAMOGE, 1999. Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens, p. 40.
Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.
Van Dyk, J.C., Pieterse, G.M., Van Vuren, J.H.J., 2007. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after the exposure to cadmium and zinc. Ecotoxicol. Environ. Saf. 66, 432–440.
Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comparat. Biochem. Physiol. 146C, 281–300.
Vieira, L.R., Sousa, A., Frasco, M.F., Lima, I., Morgado, F., Guilhermino, L., 2008. Acute effects of Benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). Sci. Total Environ. 395, 87–100.
Vieweg, I., Bilbao, E., Meador, J.P., Cancio, I., Bender, M.L., Cajaraville, M.P., Nahrgang, J., 2018. Effects of dietary crude oil exposure on molecular and physiological parameters related to lipid homeostasis in polar cod (*Boreogadus saida*). Comparat. Biochem. Physiol. 206–207C, 54–64.
Wessel, N., Menard, D., Pichavant-Rafini, K., Ollivier, H., Le Goff, J.-M., Burgeot, T., Akcha, F., 2010. The toxicity of benzo[a]pyrene on sole (*Solea solea*) hepatocytes: assessment of genotoxic and enzymatic effects. Polycycl. Aromat. Comp. 30 (5), 346–354.
Willford, W.A., 1967. Resource Publication 37. Toxicity of Dimethylsulphoxide (DMSO) to Fish. US Department of Interior.
Wu, J., Zhiming, Y., Xiuxian, S., You, W., 2006. Response of integrated biomarkers of fish (*Lateolabrax japonicus*) exposed to benzo[a]pyrene and sodium dodecylbenzene sulfonate. Ecotoxicol. Environ. Saf. 65, 230–236.
Yen, J., Donerly, S., Levin, E.D., LinneyE, A., 2011. Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. Neurotoxicol. Teratol. 33, 735–741.

Zhang, Y., Wu, R.S.S., Hong, H.-S., Poon, K.-F., Lam, M.H.W., 2000. Field study on desorption rates of polynuclear aromatic hydrocarbons from contaminated marine sediment. Environ. Toxicol. Chem. 19, 2431–2435.
Zorita, I., Ortiz-Zarragoitia, M., Apraiz, I., Cancio, I., Orbea, A., Soto, M., Marigómez, I., Cajaraville, M.P., 2008. Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using red mullets as sentinel organisms. Environ. Pollut. 153, 157–168.
Zorita, I., Cuevas, N., 2014. Protocol for fish disease assessment in marine environmental monitoring using common sole (*Solea solea*, Linnaeus 1758) as sentinel organism: identification of externally visible diseases and liver histopathology. Rev. Investig. Mar. 21, 1–18.