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Histological biomarkers in liver and gills of juvenile *Solea senegalensis* exposed to contaminated estuarine sediments: A weighted indices approach

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ABSTRACT

Young juvenile *Solea senegalensis* were exposed to three sediments with distinct contamination profiles collected from a Portuguese estuary subjected to anthropogenic sources of contamination (the Sado estuary, western Portugal). Sediments were surveyed for metals (cadmium, chromium, copper, nickel, lead and zinc), a metalloid (arsenic) and organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls and a pesticide, dichloro-diphenyl-trichloroethane plus its metabolites), as well as total organic matter, redox potential and particle fine fraction. The fish were exposed to freshly collected sediments in a 28-day laboratorial assay and collected for histological analyses at days 0 (T_0), 14 (T_{14}) and 28 (T_{28}). Individual weighted histopathological indices were obtained, based on presence/absence data of eight and nine liver and gill pathologies, respectively, and on their biological significance. Although livers sustained more severe lesions, the sediments essentially contaminated by organic substances caused more damage to both organs than the sediments contaminated by both metallic and organic contaminants, suggesting a possible synergistic effect. Correlation analyses showed that some alterations are linked, forming distinctive histopathological patterns that are in accordance with the severity of lesions and sediment characteristics. The presence of large eosinophilic bodies in liver and degeneration of mucous cells in gills (a first-time described alteration) were some of the most noticeable alterations observed and were related to sediment organic contaminants. Body size has been found to be negatively correlated with histopathological damage in livers following longer term exposures. It is concluded that histopathological indices provide reliable and discriminatory data even when biomonitoring as complex media as natural sediments. It is also concluded that the effects of contamination may result not only from toxicant concentrations but also from their interactions, relative potency and sediment characteristics that ultimately determine bioavailability.

1. Introduction

An increasing amount of research is now incorporating histopathological biomarkers in practical ecological risk assessment methodologies. Histopathological analysis has already been tested and proposed as an efficient and sensitive tool to the monitoring of fish health and environmental pollution in natural water bodies (Teh et al., 1997; Handy et al., 2002; Wester et al., 2002; Stentiford et al., 2003). The growing number of studies on histopathological biomarkers is linked to the notion that they reflect fish health more realistically than biochemical biomarkers and can thus be better extrapolated to community- and ecosystem-level effects of toxicity (Au, 2004).

Classical, essentially qualitative histopathological approaches have provided vital information on the description of histological lesions and alterations in field-collected or tested aquatic organisms (e.g. Baumann, 1985; Köhler, 1990). Nevertheless, the absence of numerical data makes it difficult to establish cause–effect relationships between pathology and contamination patterns and to assess the significance of the differences between surveyed groups. For such reason, current research on histopathological traits of exposed animals is now focusing on histopathological indices to provide numerical data based on a semi-quantitative approach. Some of these approaches have successfully employed multivariate statistics using lesion frequency indices to compare contaminated sites in biomonitoring studies (e.g. DelValls et al., 1998; Riba et

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al., 2004, 2005). Other authors have demonstrated the usefulness of semi-quantitative ranking indices based on lesion progression in several fish organs, with the advantage of providing individual indices (e.g. Schwaiger, 2001; Van Dyk et al., 2007; Triebskorn et al., 2008).

One of the most important difficulties of histopathological studies in fish relates to the lack of specificity of lesions and alterations towards a contaminant or class of contaminants, which greatly impairs cause–effect assessments when multiple toxicants are involved. On the other hand, tissue-level pathologies are by far better described in human biomedicine than in ichthyology and discrepancies in terminology and identification of lesions often arise. In an attempt to solve this issue, research is being performed in order to provide guidelines on the histopathological endpoints of exposure to xenobiotics (e.g. Koehler, 2004). Another endpoint under development concerns the actual biological significance of the analyzed lesions. Some authors now propose that condition indices should consider the relative importance of lesions since some alterations may imply greater injury to an organ than others. Weighted indices have been developed in order to fulfil this gap by attributing an ordinal-ranked value to a specific lesion according to its impact to the fish (Bernet et al., 1999).

The choice of the target organisms is also a critical factor in environmental monitoring. Due to their increased sensitivity to environmental contaminants and severity of effects on development, as well as the consequences to ecosystems and marine resources, many toxicological studies have focused on early life stage fish (Rolland, 2000). Histopathological analyses have, for instance, been successful in the assessment of the effects of organochlorine pesticides on organ development in fish larvae (Oliva et al., 2008) and hepatic lesions in juvenile fish exposed to PAHs, PCBs and organochlorines (Metcalfe et al., 1990). On the other hand, flatfish (including *Solea senegalensis*) have been successfully employed in field surveys (Simpson et al., 2000; Stentiford et al., 2003) or laboratorial exposures to sediments (Riba et al., 2004, 2005; Jiménez-Tenorio et al., 2007; Costa et al., 2008) and waterborne xenobiotics (Arellano et al., 1999; Grinwis et al., 2000).

The Senegalese sole, *S. senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae), is a common flatfish in the Sado estuary, where it is an important resource, or at least a valuable by-catch, for local fisheries. This benthic fish inhabits estuaries especially as breeding and nursing grounds, occupying sandy or muddy bottoms where it feeds on small invertebrates (Cabral and Costa, 1999; Cabral, 2000). It is a cosmopolitan species on the Atlantic coast of the Iberian Peninsula and an important aquaculture species (Dinis et al., 1999). Its ecological characteristics and ready availability (either from the field or from mariculture facilities) contribute to the species' potential as a sentinel organism for the biomonitoring of estuarine sediment contamination.

The Sado estuarine basin (Western Portugal) is a large confined coastal area subjected to various sources of anthropogenic contamination, ranging from the urban effluents from the city of Setúbal to industrial discharges from its dense heavy-industry belt. Run-offs from the extensive agricultural grounds located upstream also contribute to the transport of xenobiotics (such as pesticides and fertilizers) to the Sado basin. The estuary is an important port area and is frequently subjected to dredging to expand wharfs and to maintain navigation channels. Aquaculture and fisheries are also very important activities in the area, as well as tourism, and part of the estuary is classified as a natural reserve area. The conflict between exploitation and the need to safeguard environmental quality enhances the importance of biomonitoring studies in the estuary.

The main goals of the present work were to: (i) assess lesions and alterations on gills and livers of juvenile *S. senegalensis* exposed to sediments from three distinct stations of the Sado estuary; (ii) derive weighted histopathological condition indices; and (iii) investigate the relation between indices, lesion frequencies and sediment contaminants using a wide range of statistical analyses.

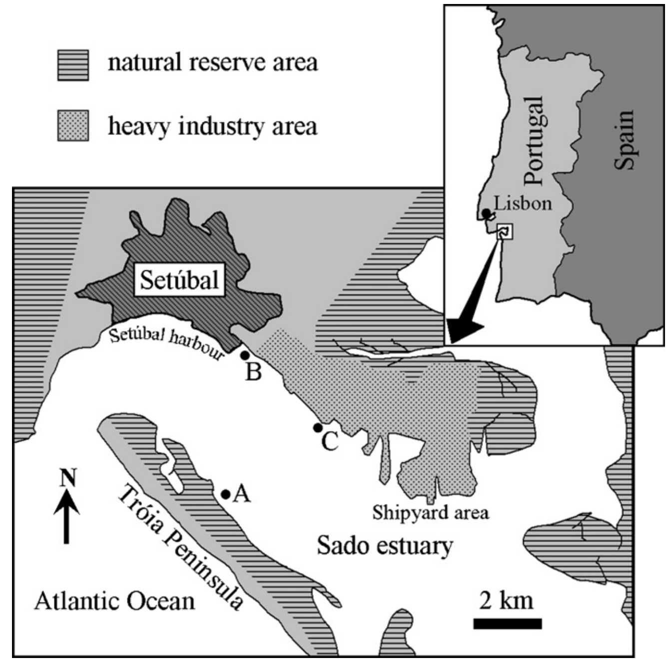

Fig. 1. Map of the study area showing the three sediment collection sites [A, B and C](•).

2. Materials and methods

2.1. Experimental assay

The tested sediments were collected with a Petit Ponar grab on November 2006 from three distinct sites of the Sado estuary (Fig. 1). Site A (the least contaminated) is the closest to a natural reserve area, the farthest from possible contamination sources and has the shortest water residence time. Sites B and C, located off Setúbal's harbour or the city's industrial belt, respectively, are potentially the most contaminated, although with different levels of contamination by metallic and organic toxicants. After collection, sediments were homogenized, transported under controlled temperature to the laboratory and were subdivided and frozen for analyses (refer to following section) or preserved at 4 °C for no longer than 5 days before the beginning of tests. For simplification purposes, exposure to the three sediments is throughout referred to as sediment tests A, B and C.

The experimental 28-day assay consisted of a closed-system recirculation arrangement of 15 L-capacity polyvinyl tanks with smooth edges to which 2 L of sediment and 12 L of clean seawater were allocated. The assay was performed in duplicate. Sediments (occupying a surface of $\approx$525 cm^2 in the tanks) were allowed to settle for 48 h before the beginning of the assay. Aeration was constant and water flow was adjusted in order to eliminate hydrodynamically driven sediment resuspension. A weekly water change (25% of total water volume) was performed in order to mimic and keep constant the animals' rearing conditions while ensuring minimal removal of potential waterborne contaminants or suspended particles and minimal stress to the fish in the test tanks. Water parameters were monitored weekly, just prior to water changes and were found to be the same as in rearing: pH 7.9 ± 0.2, salinity = 33 ± 1 g L^{-1}, temperature = 18 ± 1 °C, dissolved O_2 ranged between 40 and 45% and total ammonia within 2–4 mg L^{-1}. Photoperiod was set at 12:12 h light:dark.

Twenty four randomly selected juvenile hatchery-brood and laboratory-reared *S. senegalensis* (69 ± 6 mm standard length), all from the same cohort, were allocated to each tank. Fish were fed daily with M2 grade commercial fish pellets (AQUASOJA, Ovar, Portugal) throughout the assay. Twelve individuals (six per replicate) from each sediment test were collected per sampling time, scheduled for days 0 (T_0), 14 (T_{14}) and 28 (T_{28}) and immediately processed for histological analyses. T_0 animals consisted of twelve individuals collected directly from the rearing tanks.

2.2. Sediment analyses

The redox potential (Eh) of the sediments was determined immediately after collection using an Orion model 20A apparatus equipped with a H3131 Ag/AgCl reference electrode. Sediments were characterized for total organic matter (TOM) by complete ignition at 500 ± 50 °C. Fine fraction (FF, particle size < 63 μm) was determined by hydraulic sieving after removal of organic matter with H_2O_2, washing and disaggregation in pyrophosphate. Fine fraction and TOM are described as a percentage relatively to sediment dry weight (dw).

Trace elements were quantified from dried samples completely mineralized with a mixture of acids (6 mL HF 40%, v/v to which was added 1 mL of the mixture 36% HCl plus 60% HNO_3 3:1 v/v) for 1 h at 100 °C in closed Teflon vials, evaporated to dryness and redissolved in HNO_3 before elution in Milli-Q grade ultrapure water (Caetano et al., 2007). Arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) were quantified by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X-Series spectrometer. MESS-2 (NRC, Canada), PACS-2 (National Research Council, Canada) and MAG-1 (USGS, USA) reference sediments were analyzed by the same procedure to validate the procedure and the obtained metal concentrations were found to be within the certified range. Results are given in $\mu g\,g^{-1}$ sediment dw.

Sediment PAHs were analyzed as described by Martins et al. (2008). Briefly: dry sediment samples were spiked with surrogate standards, Soxhlet-extracted with an acetone + hexane (1:1, v/v) mixture and quantified by comparison with the retention time of standard by gas chromatography–mass spectrometry (GC–MS) using a Finnigan GCQ system. A total of seventeen 3- to 6-ring PAHs were quantified. tPAH means the sum of all individual PAHs. PCBs (18 congeners) and DDTs (pp′DDT plus metabolites: pp′DDD and pp′DDE) were quantified from dried sediment samples Soxhlet-extracted with n-hexane, fractioned in a chromatographic column and quantified by GC–MS with a Hewlett-Packard 6890 gas chromatograph (Ferreira et al., 2003). tPCB and tDDT mean the sum of all quantified PCB congeners and DDT plus metabolites, respectively. Validation was obtained by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA) and the concentrations of surveyed organic compounds were found within the certified range. Concentrations of sediment organic contaminants are expressed as $ng\,g^{-1}$ sediment dw.

2.3. Sample preparation for histological analyses

Animals were anaesthetized on ice after collection, measured for standard length (L_s) and total wet weight (ww_t) and sacrificed by cervical sectioning. Dissection was performed immediately and samples were prepared for histological analyses essentially according to Martoja and Martoja (1967). In brief: liver samples and the first and second gill arches (from the eyed side) were excised and immediately placed in Bouin-Hollande fixative (10%, v/v formaldehyde and 7%, v/v acetic acid to which picric acid was added to saturation), where they remained for 48 h (at room temperature). Samples were afterwards washed for 24 h to remove excess picric acid in a bath of distilled water (liver) or a 6% (v/v) formic acid solution in distilled water to promote decalcification (gills). Samples were afterwards dehydrated in a progressive series of ethanol dilutions and embedded in paraffin (xylene was used for intermediate impregnation). Sections (2–3 μm thick) were stained with haematoxylin and counterstained with alcoholic eosin (H&E stain) for structural analysis of gills and liver. Gill sections were also stained with alcian blue for the detection of mucosubstances (such as mucopolysaccharides and sialomucin glycoproteins) and counterstained with nuclear fast red (AB&NFR stain). Slides were mounted with DPX resinous medium (from BDH, Poole, England).

Slides were prepared in duplicate for each organ and staining procedure, with 6–8 sections per slide. A blind review of slides was performed at the end of analyses to confirm the accuracy in identification of histological traits. A DMLB model microscope (Leica Microsystems) was used for all analyses. Image analysis was performed with the software ImageJ 1.4 (Wayne Rasband National Institute of Health, Bethesda, MD, USA).

2.4. Histopathological condition indices

Histopathological condition indices for liver and gills were essentially adapted from Bernet et al. (1999). For each alteration an importance factor, or condition weight (w), was assigned, as proposed by Bernet and co-workers, based on the biological significance of the lesion, i.e. the degree in which a lesion may affect the normal functioning of a tissue or organ. Accordingly, two histopathological indices (I) were calculated: I_l (for liver) and I_g (for gills). The indices were obtained for each individual and were calculated by the simple formula:

$$I = \sum_{j=1}^{n} w_j o_j \qquad (1)$$

where w_j is the relative weight of the j-th condition and o_j a Boolean variable that assumes the values: 1 (observed) or 0 (unobserved). n is the total number of pathologies analyzed in the organ. The indices are, therefore, cumulative and account for not only the number of alterations observed in each individual but also their relative importance. Only persistent pathologies within an organ were scored as observed ($o_j = 1$), meaning that point alterations that did not qualify as representative of the overall organ condition (e.g. one necrotic cell observed in an entire liver portion or two fused lamellae in a gill arch) were disregarded, being considered as natural variations. Identification of histopathological alterations was primarily based on Hibyia (1982) and Arellano et al. (1999, 2004).

2.5. Statistical analyses

Statistics were based on the individual I values and comprised analysis of variance by means of the F-test (parametric) to assess overall differences between tests. Pairwise comparisons were obtained with the Tukey's Honest Significant Differences test (HSD test, parametric). Parametric statistics were employed after validation of the homogeneity of variances (through the Levene's test) and normality of residuals (by the Kolmogoroff–Smirnoff test). Non-parametric statistics (Kruskall–Wallis ANOVA by ranks H and Mann–Whitney U test) were performed when at least one of these assumptions was not met. Cluster analysis was based on correlation matrices by computing the Pearson's r statistic. Pairwise correlations were obtained through the Spearman's rank-order correlation ρ. The significance level was set at $\alpha = 0.05$. Statistical analysis was conducted according to Sheskin (2000) and Zar (1998) and was performed with the Statistica 6.0 software package (Statsoft Inc., Tulsa, OK, USA).

Table 1
General characterization of tested sediments.

		Site		
		A	B	C
Sediment parameters	TOM (%)	3.2	11.8	7.7
	FF (%)	37.3	97.9	76.8
	Corrected Eh (mV)	−233	−290	−316
Metallic (mg kg^{-1} sediment dw)				
	As	7.25 ± 0.15	27.43 ± 0.55	12.38 ± 0.25
	Cd	0.04 ± 0.00	0.22 ± 0.00	0.15 ± 0.00
	Cr	24.20 ± 0.48	76.33 ± 1.53	21.85 ± 0.44
	Cu	22.57 ± 0.45	167.32 ± 3.35	41.18 ± 0.82
	Ni	12.97 ± 0.26	33.67 ± 0.67	9.03 ± 0.18
	Pb	23.70 ± 0.47	66.49 ± 1.33	45.17 ± 0.90
	Zn	147.48 ± 2.95	312.23 ± 6.24	87.75 ± 1.76
Organic (ng g^{-1} sediment dw)				
PAH				
3-ring	Acenaphthene	1.41 ± 0.24	9.42 ± 1.60	4.19 ± 0.71
	Acenaphthylene	0.24 ± 0.04	1.83 ± 0.31	1.95 ± 0.33
	Anthracene	1.03 ± 0.17	10.60 ± 1.	15.34 ± 2.61
	Fluorene	1.32 ± 0.22	8.70 ± 1.48	8.03 ± 1.37
	Phenanthrene	7.96 ± 1.35	50.77 ± 8.63	54.09 ± 9.20
4-ring	Benz(a)anthracene	4.53 ± 0.77	64.60 ± 10.98	86.52 ± 14.71
	Chrysene	2.20 ± 0.37	28.31 ± 4.81	37.19 ± 6.32
	Fluoranthene	18.05 ± 3.07	170.80 ± 29.04	184.30 ± 31.30
	Pyrene	14.66 ± 2.49	131.74 ± 22.40	171.39 ± 29.14
5-ring	Benzo(a)pyrene	7.56 ± 1.28	69.81 ± 11.87	85.88 ± 14.60
	Benzo(b)fluoranthrene	6.77 ± 1.15	60.86 ± 10.35	70.25 ± 11.94
	Benzo(e)pyrene	5.12 ± 0.87	56.73 ± 9.64	62.76 ± 10.67
	Benzo(k)fluoranthrene	4.16 ± 0.71	32.21 ± 5.48	40.18 ± 6.83
	Dibenzo(a,h)anthracene	0.74 ± 0.13	7.45 ± 1.27	6.99 ± 1.19
	Perylene	4.69 ± 0.80	86.97 ± 14.79	209.16 ± 35.56
6-ring	Benzo(g,h,i)perylene	1.12 ± 0.19	39.12 ± 6.65	10.44 ± 1.78
	Indeno(1,2,3-cd)pyrene	4.87 ± 0.83	52.44 ± 8.91	51.82 ± 8.81
	tPAH	86.42 ± 14.69	882.37 ± 150.00	1 100.48 ± 187.08
PCB				
Trichlorinated	CB-18	0.04 ± 0.01	0.08 ± 0.01	0.09 ± 0.01
	CB-26	0.05 ± 0.01	0.06 ± 0.01	0.09 ± 0.01
	CB-31	0.64 ± 0.11	0.19 ± 0.03	<d.l.
Tetrachlorinated	CB-44	0.05 ± 0.01	0.38 ± 0.06	<d.l.
	CB-49	0.04 ± 0.01	0.08 ± 0.01	0.36 ± 0.06
	CB-52	0.05 ± 0.01	0.12 ± 0.02	0.45 ± 0.08
Pentachlorinated	CB-101	0.04 ± 0.01	0.23 ± 0.04	1.18 ± 0.20
	CB-105	0.03 ± 0.01	0.22 ± 0.04	0.66 ± 0.11
	CB-118	<d.l.	1.04 ± 0.18	4.92 ± 0.84
Hexachlorinated	CB-128	0.01 ± 0.00	0.08 ± 0.01	<d.l.
	CB-138	0.12 ± 0.02	0.68 ± 0.12	2.68 ± 0.46
	CB-149	0.11 ± 0.02	<d.l.	<d.l.
	CB-151	0.05 ± 0.01	0.17 ± 0.03	1.15 ± 0.20
	CB-153	0.14 ± 0.02	0.64 ± 0.11	3.39 ± 0.58
Heptachlorinated	CB-170	0.07 ± 0.01	0.27 ± 0.05	<d.l.
	CB-180	0.21 ± 0.04	0.61 ± 0.10	<d.l.
	CB-187	0.20 ± 0.03	0.72 ± 0.12	<d.l.
	CB-194	0.03 ± 0.00	0.07 ± 0.01	0.38 ± 0.06
	tPCB	1.87 ± 0.32	5.64 ± 0.96	15.34 ± 2.61
DDT				
	pp′DDD	0.10 ± 0.02	0.28 ± 0.05	0.60 ± 0.10
	pp′DDE	0.05 ± 0.01	0.27 ± 0.05	0.65 ± 0.11
	pp′DDT	0.70 ± 0.12	4.39 ± 0.75	1.18 ± 0.20
	tDDT	0.85 ± 0.14	4.94 ± 0.84	2.43 ± 0.41

FF, sediment fine fraction; TOM, sediment total organic matter; PAH, polycyclic aromatic hydrocarbon; tPAH, total PAH (sum of all individual PAHs); PCB, polychlorinated biphenyl; tPCB, total PCB (sum of all congeners); DDD, 1,1-dichloro-2,2-bis(ρ-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis (ρ-chlorophenyl)ethylene; DDT, 1,1,1-trichloro-2,2-bis (ρ-chlorophenyl)ethane; tDDT, total DDT (pp′DDD + pp′DDD + pp′DDT); <d.l., below detection limit.

3. Results

3.1. Sediment characterization

The sediments from the three sites exhibited distinct characteristics and contamination profiles (Table 1). Sediment A was found to be the least contaminated by both metallic and organic compounds and also the least anoxic sediment (i.e. with highest Eh). Sediment B presented the highest levels of all surveyed metallic contaminants and also showed the highest proportion of FF and TOM. Sediment C (the most anoxic) was essentially contaminated by organic compounds. Sediment B contained high levels of organic contaminants, especially PAHs. However, some of these substances are present in sediment C in much greater levels than those observed in B. One such compound is perylene which is present in sediment C at 241% of the concentrations observed in sediment B. Overall, the most significant PAHs were 4- and 5-ring compounds, representing ≈70% of tPAH in the three sediments. The phenanthrene/anthracene and fluoranthene/pyrene ratios were >1 and <10, respectively, for all sediments, which reflects the essentially pyrolytic origin (combustion-derived) of PAHs, as opposed to being of petrogenic origin [i.e. derived from fossil fuels (Budzinski et al., 1997)]. PCBs in sediment C were almost 3-fold compared to the levels found in sediment B, with penta- and hexachlorinated congeners representing more than 90% of tPCB. DDTs were the least represented organic toxicants, with the highest values being observed in sediment B, especially pp′DDT.

3.2. Mortality and growth

Overall mortality registered at the end of the assays was distinct between the three tests: test A caused 2% mortality whereas in tests B and C mortality was 13% and 48%, respectively. No significant differences were found between sampling times regarding fish standard length (Kruskall–Wallis *H*, $p = 0.84$) and total wet weight (Kruskall–Wallis *H*, $p = 0.97$), as well as between tests (Kruskall–Wallis *H*, $p = 0.96$ and $p = 0.70$, for length and weight, respectively). Both measurements were very significantly correlated (Spearman $\rho = 0.82$, $p < 0.01$).

3.3. Liver histopathology

The occurrence of lesions in the livers of fish collected at T_0 was low. A lesion gradient, increasing from A- to C-tested fish was clearly discernible. Lesion occurrences and severity also exhibited a tendency to increase with sampling times, for all sediment tests. Individuals collected at T_0 largely presented normal livers (Fig. 2A), exhibiting regular cells with a translucent, virtually unstained cytoplasm in which inclusions were absent. These clear-type hepatocytes observed in healthy livers stained with H&E should indicate good storage of glycogen (Simpson, 1992). Nuclei were observed to be of constant-size and shape, with well individualized nucleoli. Many sinusoids line the hepatic cords, branching from large venous vessels.

Foci of eosinophilic (acidophilic) hepatocellular alteration were found in exposed individuals of all tests but with an obvious increase in occurrences in B- and C-tested fish, especially at T_{28}. Some T_0 individuals also exhibited this non-specific pathology that is considered to be a pre-neoplastic lesion (Vethaak and Wester, 1996). These foci were occasionally found to be associated with proliferation and swelling of blood vessels (Fig. 2B). Altered cells were frequently observed to have intraplasmatic anomalies such as vacuolation derived from lipidosis and eosinophilic bodies (Fig. 2C).

Focal hepatic necrosis was observed in all tests at all sampling times except in T_0 and A-tested individuals collected at T_{14}. Nevertheless, the occurrence and extension of necrotic areas was more significant in B- and, especially, C-tested individuals, reaching the

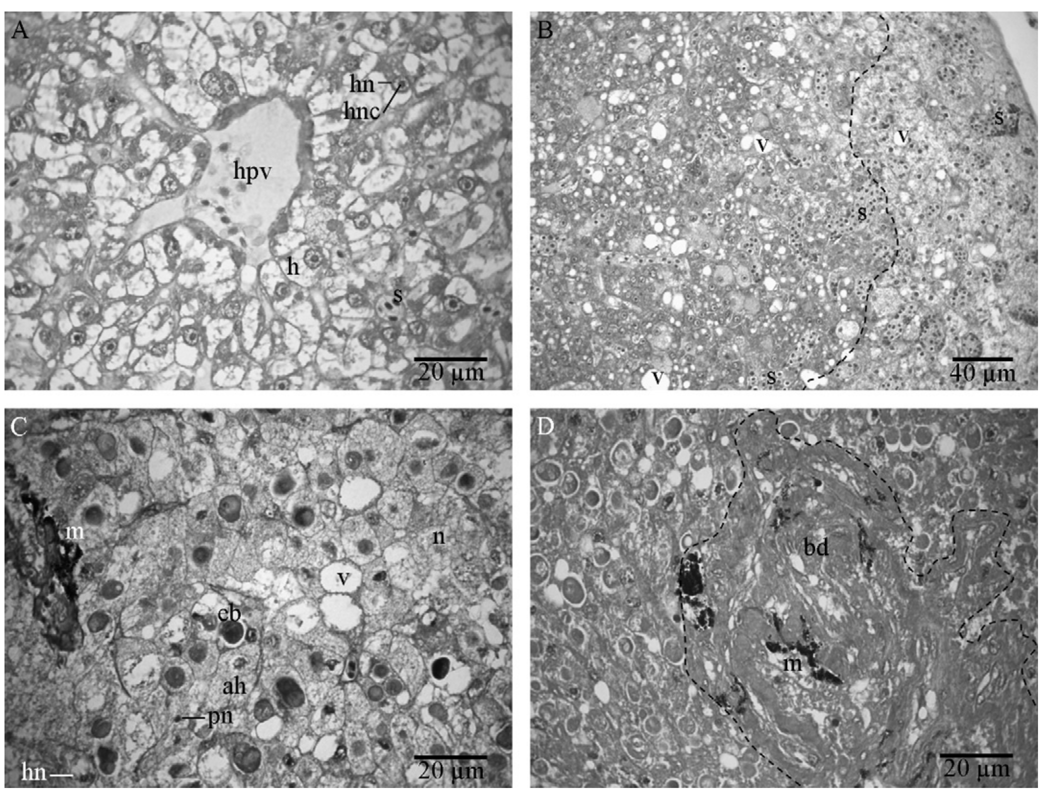

Fig. 2. Liver sections of tested individuals (H&E). (A) Normal hepatic parenchyma of a fish collected in the beginning of the assay, exhibiting well-defined hepatocytes, polyedric in shape. (h) hepatocyte; (hn) hepatocyte nucleus; (hnc) nucleolus; (hpv) hepatic portal vein branch with erythrocytes; (s) transversally sectioned sinusoid. (B) Extensive lipidosis causing proliferation of intracellular vacuole-like structures (v) and a large eosinophilic area (right to dashed line) in a fish exposed for 14 days to sediment B (contaminated by metallic and organic substances). Proliferation and swelling of sinusoids (s) are also evident. (C) Eosinophilic hepatocellular alteration in the liver of a fish exposed for 28 days to sediment C (essentially contaminated by organic compounds). Altered hepatocytes (ah) tend to retain eosin in the cytoplasm and to lose the typical polyedric shape. Melanomacrophages (m) at a vein are also evident, as well as necrotic foci (n), vacuoles (v) and eosinophilic bodies inside altered hepatocytes (eb). Many altered cells exhibit nuclear pleomorphisms [picnotic (pn) and hypertrophied (hn) nuclei]. (D) Granulomatous lesion in the liver of a fish exposed to sediment C for 28 days. The lesion (inside the dashed line) is located around a regressed bile duct (bd) and infiltrates the highly damaged surrounding tissue. Melanomacrophages (m) can be observed inside the lesion.

stage where hepatic structure was no longer discernible and tissue underwent structural rupturing (Fig. 2C). Melanomacrophages were often observed in necrotic areas. Eosinophilic bodies are cytoplasmic, well-delimited, reddish inclusions commonly observed in association with strongly damaged tissue (Fig. 2C). Under H&E stain these inclusions retain a strong red pigmentation (from eosin). The presence of eosinophilic bodies is occasionally termed hyaline degeneration. The presence of these inclusions was most prominent in C-test animals. Eosinophilic bodies appeared to be membrane-delimited, ellipsoidal in shape and were present in small numbers inside the cells, usually one or two. These inclusions were variable in size. Although close to the adopted α, 'no statistical differences (Mann–Whitney *U*, $p = 0.07$) was found between the length of the largest axles of eosinophilic bodies of T_{14} and T_{28} C-tested fish ($3.3 \pm 1.7\ \mu m$ and $5.7 \pm 0.6\ \mu m$, respectively).

Foci of unspecified granulomatous lesions were occasionally observed in the livers of C-tested individuals. These lesions consisted of foci of highly degenerated tissue where melanomacrophages were discernible, without having been observed to form dense centres (Fig. 2D).

3.4. Gill histopathology

As opposed to what was observed in livers, more than half of T_0 individuals (i.e. collected from the rearing tanks) showed moderate gill damage (Fig. 3A). Individuals tested with sediments B and C were found to suffer the most severe lesions. Sediment particles were not observed on lamellae and interlamellar spaces. No ecto- or endoparasites were observed.

A moderate hyperplasia of interlamellar epithelial cells was often observed, occasionally originating foci of lamellar fusion (but not rod-shaped filaments), particularly in C-tested individuals (Fig. 3B). Chloride cell hypertrophy was a also frequent lesion in B- and C-tested individuals (Fig. 3B). This type of damage provided gill epithelia with a vacuolated appearance since hypertrophied chloride cells enlarge and gain a vacuole-like appearance, indicating a possible fluid retention. The mean length of the largest diameter of normal chloride cells was $9.2 \pm 1.1\ \mu m$ (of T_0 fish) and that of hypertrophied was $14.7 \pm 4.2\ \mu m$ (of B- and C-tested individuals). The difference between the two forms was found to be statistically significant (Mann–Whitney *U*, $p < 0.05$). No obvious change in the number of chloride cells was observed.

The fish exposed to sediment C frequently presented circulatory disturbances, the most recurrent of which was the swelling of the apical vessels of lamellae due to blood congestion [also termed aneurysm or telangiectasia (Fig. 3C)]. In addition, several types of structural deformities in gill lamellae were observed (Fig. 3D), especially in B- and C-tested fish, and the most damaged gills often presented severe hypertrophy and shedding of squamous epithelia cells (desquamation).

Mucous (goblet) cells suffered a very clear regression, in number and size, almost exclusively in C-tested individuals, as revealed by the alcian blue test (Fig. 4). This alteration was observed in all C-tested fish collected at T_{28}. Degeneration of goblet cells was visible in the entire organ (not limited to occasional foci) and was accompanied by an absence in secreted mucous between lamellae, whereas secreted mucous was observed between the lamellae of gills with normal goblet cells. The largest diameter of normal goblet cells (of T_0 individuals) measured $9.3 \pm 1.5\ \mu m$, whereas that of the atrophied cells (of C-tested fish) was $5.2 \pm 1.5\ \mu m$. Significant differences were found between the measurements of normal and atrophied cells (Mann–Whitney *U*, $p < 0.01$).

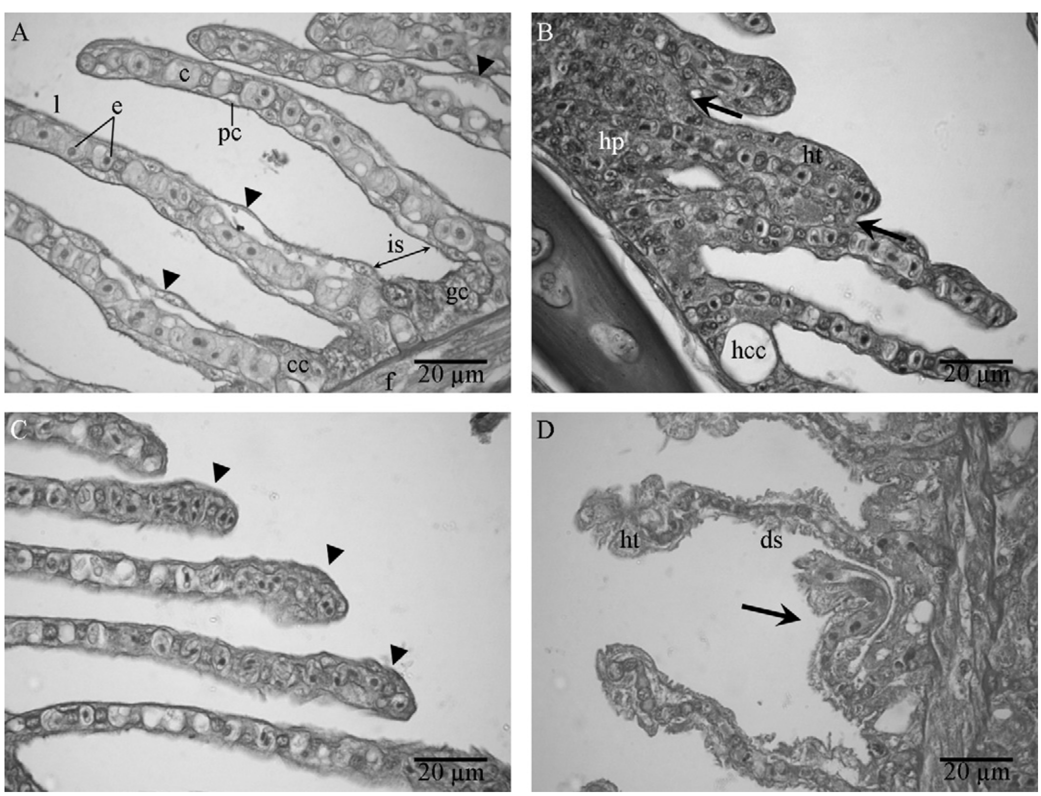

Fig. 3. Gill sections of tested fish (H&E) (A) Gill from an individual collected in the beginning of the assay, where the only visible alteration is a minor lifting of squamous epithelia (arrowhead). (c) blood capillary; (cc) chloride cell; (e) erythrocyte in capillary; (f) gill filament; gc) goblet (mucous secreting) cell; (is) interlamellar space; (l) gill lamella. (B) Gills of a fish after 14 days of exposure to sediment C (mostly contaminated by organic substances), exhibiting epithelial hyperplasia (hp) and hypertrophy (ht), ultimately leading to fusion of lamellae (arrow). (hcc) hypertrophied chloride cell. (C) Circulatory disturbances in terminal vessels of lamellae (arrowheads) in a fish exposed to sediment C for 14 days. Erythrocyte accumulation in capillaries and swelling of lamellar tips are evident. (D) Deformed gill lamella (arrow) of a fish exposed to sediment C for 28 days. Desquamation (ds) and hypertrophia (ht) of lamellar epithelial cells are also evident.

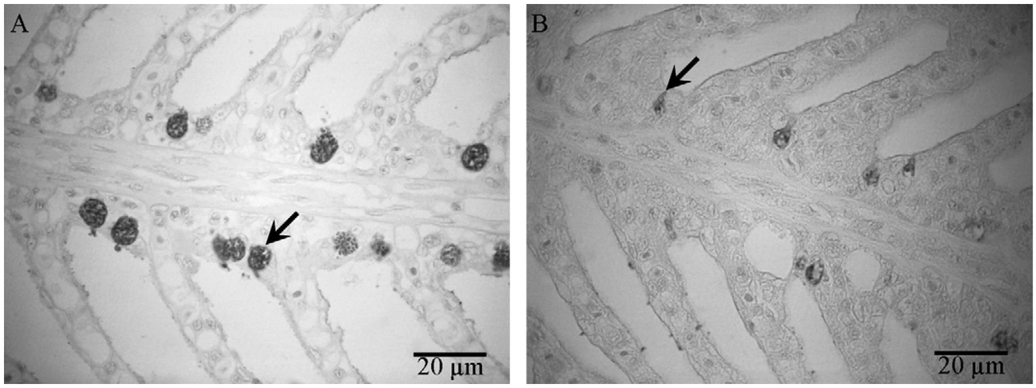

Fig. 4. Mucous (goblet) cells (arrows) in the gills of individuals after 28 days of exposure to tested sediments (AB&NFR). (A) Normal morphology of goblet cells in an individual exposed to the reference sediment (sediment A). (B) Atrophied goblet cells in a fish exposed to essentially organic-contaminated sediments (sediment C).

3.5. Histopathological condition weights

A list of all observed pathologies and respective condition weights is presented in Table 2. The condition weights were essentially adopted from Bernet et al. (1999), ranging between $w = 1$ and $w = 3$: Necrosis has the maximum value ($w = 3$), followed by granulomatous lesions, hyperplasia, cell atrophy and genetic material-related alterations such as nuclear pleomorphisms (picnosis and hypertrophy), with $w = 2$. Structural changes in cells and tissues (like hepatocyte vacuolation, squamous cell hypertrophy or lamellar deformation in gills), inflammatory responses (such as blood vessel swelling and presence of melanomacrophages) were given the lowest value ($w = 1$). No specific information exists regarding the biological significance of the presence of eosinophilic bodies in fish cells but some biomedical and pathological research has linked this non-specific alteration to severe lesions such as hepatic neoplasms (Chedid et al., 1999). For this reason, a condition weight $w = 2$, equal to eosinophilic cellular alteration, was attributed to the presence of eosinophilic bodies. Similarly, little is known about the real significance of chloride cell hypertrophy. Results from exposure to metals indicate that alterations such as hypertrophy and proliferation of these cells have a very important effect on the thickening of epithelia and ion exchange processes, therefore impairing respiration and osmotic balance (Mazon et al., 2002). For this reason, chloride cell hypertrophy in the gills has been given a $w = 2$ value, whereas squamous cell hypertrophy retains the $w = 1$ value proposed by Bernet et al. (1999). Atrophy of gill mucous cells is a first-time described lesion in the present study. Due to the possible severe consequences caused by a deficiency in mucous secretion, which acts as the gill's primary defence barrier to the environment of the animal, it has been attributed a $w = 2$ value.

Table 2
Observed pathologies and their condition weights (*w*).

Target organ	Reaction pattern	Alteration	*w*
Liver	Inflammatory response	Profusion and dilation of blood vessels	1
		Presence of melanomacrophages	1
	Regressive	Nuclear pleomorphisms	2
		Necrosis	3
	Progressive	Lipidosis	1
		Presence of eosinophilic bodies	2
		Eosinophilic hepatocellular alteration	2
		Granulomatous lesions	2
Gills	Circulatory disturbances	Lamellar capillary aneurism (telangiectasia)	1
	Regressive	Epithelial lifting	1
		Epithelial desquamation	1
		Deformation of lamellae	1
		Mucous (goblet) cell degeneration	2
	Progressive	Hypertrophy of squamous epithelia	1
		Lamellar fusion	1
		Chloride cell hypertrophy	2
		Epithelial hyperplasia	2

3.6. Histopathological condition indices

The two indices were found to be significantly correlated (Spearman $\rho = 0.60$, $p < 0.01$). In general, both indices revealed highly significant differences between tests and sampling times (*F* test, $p < 0.01$). The test with sediment C revealed the most significant differences from T_0 regarding I_l and I_g at both sampling times, followed by test B, but only for I_l. Test A did not cause a significant increase of either indices in relation to T_0 (Fig. 5). I_l from C-tested individuals collected at T_{28} differed significantly from A- and B-tested fish (Tukey HSD, $p < 0.05$). No significant differences were found between T_{28} and T_{14} I_l and I_g, for all tests, although a statistical difference close to the significance threshold was observed between the I_ls of C-tested fish collected at T_{14} and T_{28} (Tukey HSD, $p = 0.07$). Regarding gill indices, only C-tested fish showed significant differences from T_0 individuals, but no such differences were found in other tests, or between sampling times (Tukey HSD, $p > 0.05$).

Cluster analyses derived correlations between lesions (Fig. 6). Regarding hepatic lesions, three groups of lesions are conspicuous. The first group comprises granulomatous lesions and the presence

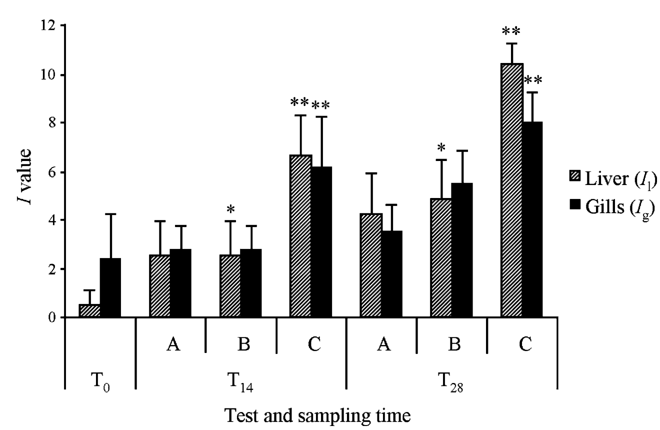

Fig. 5. Mean values of the histopathological condition indices (*I*) obtained from liver and gills of tested individuals. (* and **) Indicate significant differences from T_0, $p < 0.05$ and $p < 0.01$, respectively (Tukey's HSD test). Error bars represent 95% confidence intervals.

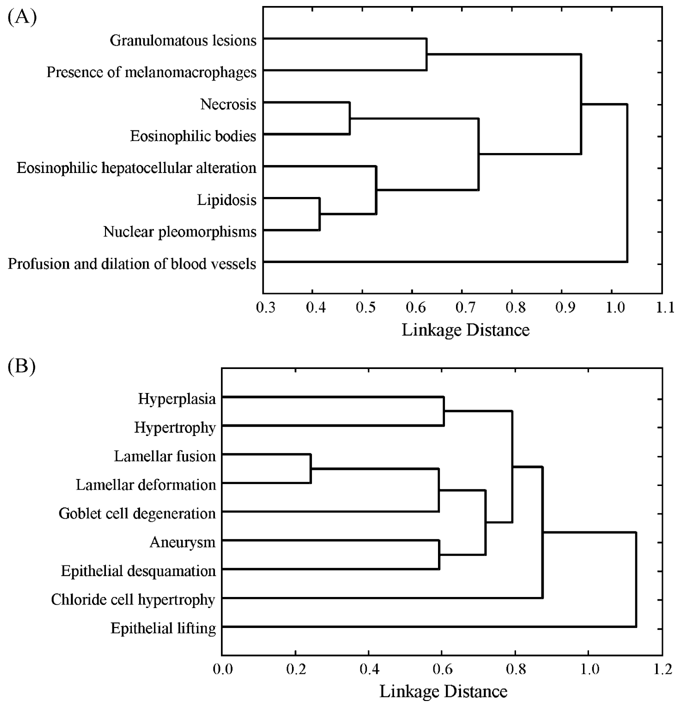

Fig. 6. Joining tree of observed lesions in liver (A) and gills (B) of exposed individuals. Distances were obtained from presence/absence data and estimated as 1-Pearson *r*. Joining is based on unweighted pair-group averages.

Table 3
Correlation analyses between condition indices and sediment contaminants plus growth variables.

Sampling time		I_l		I_g	
		Spearman ρ	*p*-Level	Spearman ρ	*p*-Level
T_{14}	As	0.60	0.00	0.42	0.03
	Cd	0.60	0.00	0.42	0.03
	Cr	–	n.s.	–	n.s.
	Cu	0.60	0.00	0.42	0.03
	Ni	–	n.s.	–	n.s.
	Pb	0.60	0.00	0.42	0.03
	Zn	–	n.s.	–	n.s.
	tPAH	0.62	0.00	0.51	0.01
	tPCB	0.62	0.00	0.51	0.01
	tDDT	0.60	0.00	0.42	0.03
	L_s	–	n.s.	–	n.s.
	ww_t	–	n.s.	–	n.s.
T_{28}	As	–	n.s.	0.40	0.04
	Cd	–	n.s.	0.40	0.04
	Cr	−0.63	0.00	–	n.s.
	Cu	–	n.s.	0.40	0.04
	Ni	−0.63	0.00	–	n.s.
	Pb	–	n.s.	0.40	0.04
	Zn	−0.63	0.00	–	n.s.
	tPAH	0.68	0.00	0.73	0.00
	tPCB	0.68	0.00	0.73	0.00
	tDDT	–	n.s.	0.40	0.04
	L_s	−0.52	0.01	–	n.s.
	ww_t	−0.55	0.01	–	n.s.

I_l, liver condition indice; I_g, gill condition indice; L_s, fish standard length; ww_t, fish total wet weight; n.s., non-significant.

of melanomacrophages, the second necrosis and eosinophilic bodies, and the third eosinophilic hepatocellular alteration, lipidosis and nuclear pleomorphisms. It is noteworthy, though, that the aforementioned second and third groups can be grouped in a distinct category from the first, according to linkage distances. Blood vessel inflammatory responses are depicted as an independent type of alteration poorly correlated with other lesions. In gills, the strongest correlation was observed between fusion and deformation of lamellae, which, together, are linked to mucous cell degeneration. Aneurysms and epithelial desquamation are also correlated and can be placed in the same cluster of lesions as the previous, forming a distinct group from epithelial hyperplasia and hypertrophy. Chloride cell and epithelial lifting are depicted as the two alterations most detached from other lesions.

PAHs and PCBs were found to be the best correlated sediment contaminants with both condition indices, at both T_{14} and, especially, at T_{28} (Table 3). Liver indices, however, have in general better correlations with the surveyed contaminants than gills. Liver indices of individuals collected at T_{28} also depict a significant negative correlation with both growth variables and with the metals Cr, Ni and Zn, a result not observed for gill indices.

4. Discussion and conclusions

The present study demonstrated that different profiles of sediment contamination cause distinct patterns of chronic histological lesions in juvenile *S. senegalensis*. These patterns, however, have not shown a linear relationship with cumulative sediment contamination, although exposure to sediment A (the least contaminated) caused least histopathological lesions and alterations, and exposure to sediment C (mostly contaminated by organic toxicants) caused the most severe lesions in both organs, in accordance with overall mortality. A comparison between the contaminant concentrations of test sediments and some of the most commonly considered sediment quality guidelines (SQGs) for coastal areas (MacDonald et al., 1996), namely the threshold effects level (TEL) and the probable effects level (PEL), suggests that sediment B should have been responsible for the highest toxicity (Table 4). However, the overall contamination of tested sediments can be considered moderate: PEL thresholds are only reached for Cu and Zn and in sediment B only. The severity of lesions observed in test C might be explained by three factors: (1) the highest concentration in sediment C of a few organic compounds, especially some PAHs and PCBs; (2) the synergistic (rather than cumulative) effects of metallic and organic xenobiotics, which may have caused a decrease or a delay in toxicity in B-tested fish; and (3) a higher release of contaminants from sediment C than other sediments during the assay, enhancing toxicant bioavailability.

The negative correlations found between sediment metals (Cr, Ni, and Zn) and I_l at T_{28} could indicate that, at least at a later stage of exposure, metals may have an antagonistic effect with organic contaminants. The higher damage observed in fish exposed to sediment C, when compared to fish exposed to sediment B, is in accordance with this statement. This interaction between the two classes of contaminants was not observed in the gills since I_g was observed to be positively correlated to sediment contaminants, especially PAHs and PCBs, throughout the experiment. The negative correlation between I_l and body size variables (standard length and total wet weight) indicates that smaller animals may be more susceptible to hepatic chronic lesions as a result from exposure to xenobiotics.

Previous research related hepatocellular alterations such as eosinophilic foci and nuclear abnormalities in fish hepatic tissue to exposure to PAHs and PCBs (Mikaelian et al., 1998; Myers et al., 1998) and linked tissue degeneration, pre-neoplastic and neoplastic diseases to citochrome activity and PAH-DNA adducts in flatfish liver (Köhler and Pluta, 1995; Lyons et al., 2004). Detoxification of organic toxicants such as PAHs involves activity of cytochrome P450 (CYP1A) monoxygenase enzymes, to produce the more soluble but highly toxic activated forms (like PAH *o*-quinones) and reactive oxygen species (Flowers-Geary et al., 1996; Burchiel et al., 2007). Metals and metalloids, on the other hand, are known to impair CYP1A induction and activation of PAHs (Vakharia et al., 2001).

Table 4
Comparison between the concentrations of surveyed sediment contaminants and available SQGs. TEL and PEL values are given in mg kg^{-1} sediment dw for metals and μg g^{-1} sediment dw for organic substances (from MacDonald et al., 1996).

Contaminant			Site			TEL[a]	PEL[b]
			A	B	C		
Metalloid		As	>TEL	>TEL	>TEL	*7.24*	*41.6*
Metal		Cd	–	–	–	*0.68*	*4.21*
		Cr	–	>TEL	–	*52.3*	*160*
		Cu	>TEL	>PEL	>TEL	*18.7*	*108*
		Ni	–	>TEL	–	*15.9*	*42.8*
		Pb	–	>TEL	>TEL	*30.2*	*112*
		Zn	>TEL	>PEL	–	*124*	*271*
PAH	3-ring	Acenaphthene	–	>TEL	–	*6.71*	*88.9*
		Acenaphthylene	–	–	–	*5.87*	*128*
		Anthracene	–	–	–	*46.9*	*245*
		Fluorene	–	–	–	*21.2*	*144*
		Phenanthrene	–	–	–	*86.7*	*544*
	4-ring	Benz(a)anthracene	–	–	>TEL	*74.8*	*693*
		Chrysene	–	–	–	*108*	*846*
		Fluoranthene	–	>TEL	>TEL	*113*	*1494*
		Pyrene	–	–	>TEL	*153*	*1398*
	5-ring	Benzo(a)pyrene	–	–	–	*88.8*	*793*
		Benzo(b)fluoranthrene	*NG*	*NG*	*NG*	*NG*	*NG*
		Benzo(e)pyrene	*NG*	*NG*	*NG*	*NG*	*NG*
		Benzo(k)fluoranthrene	*NG*	*NG*	*NG*	*NG*	*NG*
		Dibenzo(a,h)anthracene	–	>TEL	>TEL	*6.22*	*135*
		Perylene	*NG*	*NG*	*NG*	*NG*	*NG*
	6-ring	Indeno(1,2,3–cd)pyrene	*NG*	*NG*	*NG*	*NG*	*NG*
		Benzo(g,h,i)perylene	*NG*	*NG*	*NG*	*NG*	*NG*
		tPAH	–	–	–	*1.684*	*16.770*
PCB		tPCB	–	–	–	*21.6*	*189*
DDT		pp′DDD	–	–	–	*1.22*	*7.81*
		pp′DDE	–	–	–	*2.07*	*374*
		pp′DDT	–	>TEL	–	*1.19*	*4.77*
		tDDT	–	>TEL	–	*3.89*	*51.7*

NG, no guideline available; [–], values below SQGs.
[a] TEL, threshold effects level: concentration below which contamination effects rarely occur.
[b] PEL, probable effects level: concentration above which contamination effects occur frequently.

This synergistic effect between metallic and organic contaminants may contribute to explain the reduced hepatic damage observed in B-tested fish compared to C-tested animals.

Hepatic fatty degeneration observed as lipidosis (intracellular lipid storage in large vacuoles, as opposed to steatosis caused by microvesicular lipid accumulation) was one of the most recurrent alterations found in the livers of fish exposed to sediments B and C. Hepatic lipidosis has been observed in fish exposed to metals (Arellano et al., 1999; Giari et al., 2007), crude oil extracts (Solangi and Overstreet, 1982) and in feral fish from sites contaminated by mixtures of xenobiotics (Greenfield et al., 2008; Triebskorn et al., 2008). Although some authors have discussed that lipid droplets in hepatocytes may store insoluble contaminants or their by-products (Köhler, 1990), this type of alteration has been regarded as a general failure in lipid metabolism as a result of exposure to undifferentiated xenobiotics rather than a specific response (Van Dyk et al., 2007), which is in accordance with the present observations.

The presence of large eosinophilic inclusions in hepatocytes of highly damaged livers is one of the most conspicuous alteration pattern observed. Information is missing regarding the exact causes of this alteration and its consequences to organ function. Eosinophilic bodies in flatfish liver and kidney have already been linked to the exposure to xenobiotics (Camargo and Matinez, 2007; Van Dyk et al., 2007). One of the striking differences, however, between the inclusions observed in the present study and the ones described in the literature is their size. Eosinophilic bodies mentioned in previous studies appear to be much smaller than the ones observed in individuals exposed to sediment C for 28 days. Information on the nature of the substances contained in these inclusions is absent but, considering the affinity of eosin to structural proteins such as actin, it is possible that eosinophilic bodies retain peptide material absorbed from the cytoplasm of degenerating cells. This is supported by data on eosinophilic bodies found in neoplastic areas of human epithelia (Buchner et al., 1976) and liver (Chedid et al., 1999). Considering their correlation to necrosis, eosinophilic bodies may be indicators of severe cirrhosis. Furthermore, their high frequency in the livers of fish exposed to sediment C suggests a link between eosinophilic bodies and exposure to organic contaminants. Altogether, hepatic alterations such as eosinophilic bodies, eosinophilic hepatocellular alteration and lipidosis appear to form a ubiquitous, non-specific group of histopathological alterations within vertebrates, from fish to humans.

The correlation between I_l and I_g may indicate that gills were the major entry organs of contaminants released from the sediments. In fact, no organisms or significant amounts of sediment were found in the guts of tested fish, showing that fish were feeding essentially on pellets. It is thus likely that the digestive system was not primarily involved in the uptake of xenobiotics. Exposure to metallic and organic contaminants has been linked to acute lesions in gills, like aneurisms and lamellar fusion and, simultaneously, to more severe, chronic hepatic alterations such as lipidosis and neoplastic diseases (Roberts and Oris, 2004; Oliveira Ribeiro et al., 2005). This information is in accordance with the present findings and suggests that gills are more susceptible to the immediate (acute) effects of exposure to waterborne contaminants and livers are subjected to the more prolonged (chronic) effects of accumulated contaminants and their, often more toxic, metabolites. The chronic effects observed in the livers of exposed fish, especially those of B- and C-tested fish indicate prolonged physiological disturbances that led to glycogen depletion and lipid storage, as well as hepatocellular alterations and necrosis. On the other hand, some of the most recurrent gill lesions, namely circulatory disturbances and epithelial hypertrophy and hyperplasia, are known to be reversible (Poleksić and Karan, 1999; Guimarães et al., 2007).

It should be noted that feral animals collected from sites considered to be clean often present a baseline level of non-specific gill lesions. Some authors have suggested that some of these lesions may be originated from parasitosis (Teh et al., 1997; Schwaiger, 2001; Handy et al., 2002). In the present study no indicators of parasites were found in the gills of *S. senegalensis*, which suggests that other environmental parameters may have been the cause of gill lesions observed in fish collected at T_0 (such as ammonia levels in the rearing systems). These lesions may be considered as baseline alterations and support the statement that naturally occurring histopathological damage may be an important confounding factor in biomonitoring studies.

One of the most distinctive gill lesions observed was the hypertrophy of chloride cells. Considering the resemblance of hypertrophied cells to empty-like structures, it is unlikely that this alteration was caused by an increase in the metabolic capacities of the cells, which would be reflected in proliferation of mitochondria and endoplasmatic reticuli. It is possible that these alterations cause an imbalance of osmotic regulation by impairing ionic active transport (Mazon et al., 2002). Chloride cell hypertrophy is generally regarded as a response in fish subjected to salinity changes (Karnaky et al., 1976; Foskett et al., 1981) but it has also been found to result from exposure to waterborne pesticides (Fanta et al., 2003). Since water parameters were held constant and kept similar to rearing conditions, chloride cell hypertrophy may have been caused by contaminants released from the sediments.

Another important gill lesion observed in exposed fish was the regression of mucous (goblet) cells, in both number and size. This alteration was observed almost exclusively in C-tested individuals, especially at T_{28}, when all surveyed individuals exhibited the pathology. No previous observations of this gill epithelial alteration were found in the literature. The results suggest that atrophy of mucous cells is linked to the characteristics of sediment C, especially its high concentrations of PAHs and PCBs. This alteration may have affected fish health and survival since gill mucous provides vital protection of the delicate structure and epithelia of the gills. The link found between this alteration and structural damage such as lamellar fusion and deformation substantiates this. Also, since mucosubstances act as a primary trap to exogenous substances, it is possible that damage to this barrier increased the exposure to waterborne contaminants. As for chloride cells, the differences found between the size of normal and atrophied goblet cells indicate that cell measurements may be an important quantitative biomarker.

Sediment collection and handling during the preparation of the assay, combined with the resuspension caused by the scavenging activities of the animals may have enhanced the release of contaminants into the water column. Bioturbation has been found responsible for prolonged bioavailability of the released contaminants (Atkinson et al., 2007). This release may have affected the three tests, contributing to explain some of the damage observed in individuals exposed to sediment A. However, the combination of high anoxia with intermediate FF and TOM contents with sediment resuspension is probably responsible for an increased discharge of contaminants from sediment C (Vale et al., 1998; Caetano et al., 2003; Eggleton and Thomas, 2004).

In conclusion, semi-quantitative indices based on the relative weights of lesions and quantitative data such as the eosinophilic bodies, chloride and goblet cell measurements applied in the present work provide a more biologically realistic and effective approach to analyze histopathological lesions than traditional frequency-based indices. The combination of histopathological indices with several statistical approaches made possible to correlate lesions to sediment contaminants. On the other hand, analysis of frequencies permitted a sensible grouping of histopathological lesions that is in accordance with their biological significance. In addition, laboratory exposures to natural sediments may enhance toxicity by increasing contaminant bioavailability through sediment disturbance; a relevant finding since the assays may have mimicked sediment disturbance events in estuaries such as dredgings, storms and heavy run-offs.

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