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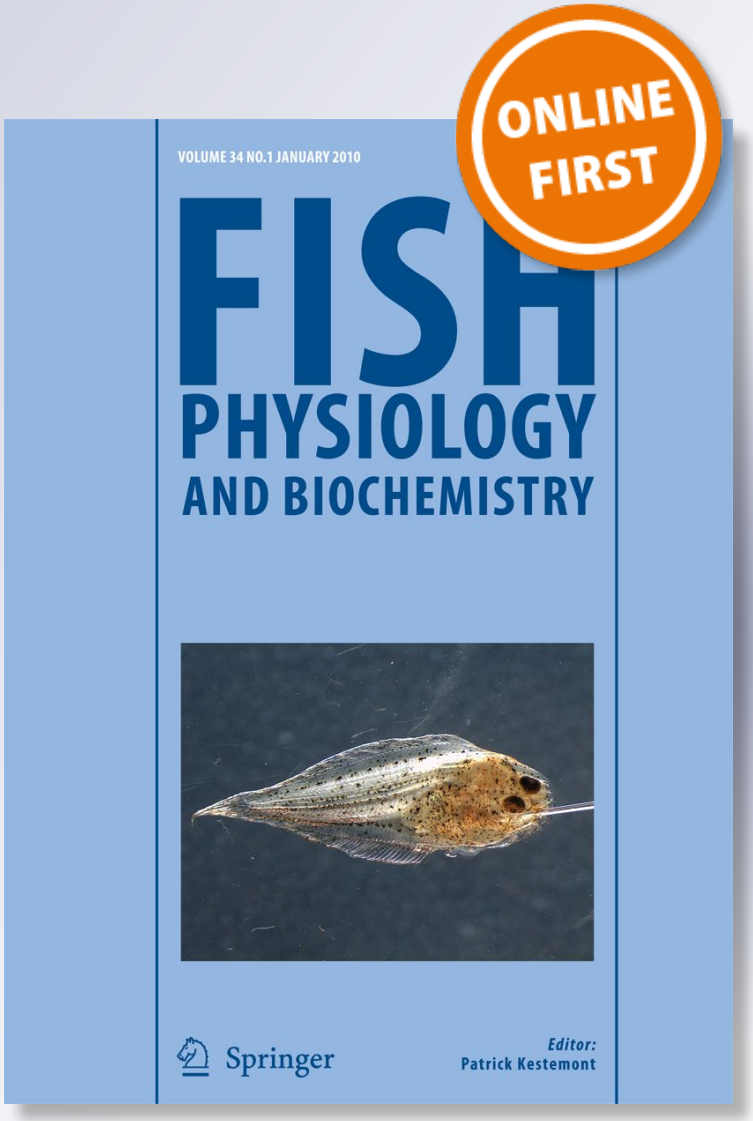

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Multi-organ histological observations on juvenile Senegalese soles exposed to low concentrations of waterborne cadmium

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Abstract A histopathological screening was performed on juvenile Senegalese soles exposed to environmentally realistic concentrations of waterborne Cd (0.5, 5 and 10 μg L^{-1}) for 28 days. The severity and dissemination of histopathological changes were variable and limited to the kidney, liver, spleen, gills and skin goblet cells. Contradicting available literature that refers the liver as the most affected organ upon acute exposure and the kidney following chronic exposure, the liver was the most impacted organ (even at the lowest concentration), in a trend that could relate to the duration of exposure and Cd concentration. The most noticeable hepatic alterations related to inflammation, although hepatocellular alterations like lipidosis and eosinophilic foci also occurred. The trunk kidney of exposed fish endured moderate inflammation, apoptosis and necrosis, however, without a clear time-dependent effect. The spleen of fish subjected to the highest concentrations revealed diffuse necrotic foci accompanied by melanomacrophage intrusion. The gills, albeit the most important apical uptake organ of dissolved toxicants, sustained only moderate damage, from epithelial hyperplasia and pavement cell detachment to the potentially more severe chloride cell alterations. In the skin, an increase in goblet cell size occurred, most notoriously correlated to Cd concentration at earlier stages of exposure. The results show that a metal-naïve juvenile fish can endure deleterious effects when exposed to low, ecologically relevant, concentrations of a common toxic metal and that the pattern of Cd-induced histopathological alterations can be complex and linked to organ-specific responses and metal translocation within the organism.

Keywords Histopathology · Metal · *Solea senegalensis* · Sub-lethal exposure · Bioassays

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Introduction

Cadmium (Cd) is a non-essential, highly toxic, metal (in either inorganic or organic species) that may contaminate the environment from natural erosion of Cd-containing minerals and soils or, most importantly, from anthropogenic sources. Cadmium toxicity is

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known since the mid-nineteenth century from workers occupationally exposed to the metal. Its severe effects to human health have been widely studied and documented ever since, from nephrotoxicity to carcinogenicity and reproductive disorders (e.g. Nordberg 2009 and Nawrot et al. 2010, for recent reviews on Cd pollution and toxicity to humans). Although its industrial use has been enduring many restrictions, this metal is still widely employed, for instance, in batteries, dyes, metal alloys and even some phosphate fertilizers, to which is added its release from the combustion of fossil fuels and from metal extraction of cadmium-containing ores. Cadmium thus reaches the marine environment chiefly through continental run-offs plus urban and industrial effluents. Estuaries and other confined coastal waterbodies are particular areas of concern due to their ability to trap, store and speciate Cd, as well as other inorganic and organic toxicants. Cadmium is normally included in biomonitoring studies with fish and other aquatic organisms. Due to its hazardous nature, Cd is classified as a Priority Substance by the Directive 2008/105/CE of the European Parliament and of the Council, known as the Water Framework Directive, which sets its highest admissible concentration at 1.5 μg L^{-1} (applicable to non-inland surface waters). In addition to its relevance as an environmental hazard, Cd has been regarded as a model toxicant of metals (as benzo[a]pyrene or tetrachlorodibenzodioxin are for organic contaminants) in many mechanistic studies (for which the toxicity of this metal per se may not be the main goal) that recur to heterodox exposure routes, from gavage to intraperitoneal injections, or may involve in vitro exposures, for example, to cell lines such as the human HepG2 (hepatocellular carcinoma) or the EPC (skin tumour) from carp and others (for instance Dayeh et al. 2005; Muylle et al. 2006; Escobar et al. 2009).

Teleosts have long been targeted in toxicological studies involving aquatic pollutants due to their ecological relevance, availability and ability to act as surrogates for higher vertebrates. These studies involve either collecting feral animals or bioassays with locally exposed (caged) animals or even laboratory bioassays to test the toxicity of single or combined substances. In either case, histopathological assessment in fish as long been recognized as a highly valuable tool to identify the toxicopathic effects of substances since it may better reflect the true health condition of the animal than other biomarker/diagnosis methods (see, e.g., van der Oost et al. 2003; Wester et al. 2002 and Au 2004). Still, fish histopathology is far to be as standardized, with respect to lesion detailing, identification and nomenclature, as in higher vertebrates (i.e. mammals, including humans), to which are added difficulties in establishing cause-effect relationships and the lack of specificity of most biomarker candidates. Furthermore, there are yet few studies with fish exposed to environmentally realistic concentrations of waterborne Cd and even fewer concerning histopathology. Giari et al. (2007), for instance, found conclusive histopathological alterations in multiple organs of *Dicentrarchus labrax* (L. 1758) exposed to waterborne Cd for 24–48 h, but the lowest concentration tested was $\approx$ 5 mg L^{-1}. However, Cd concentrations in impacted marine or brackish aquatic environments usually range up to 10 μg L^{-1} (see for instance Power et al. 1999; Waeles et al. 2004). In the few bioassay-based studies performed with fish within this range of contamination, Lizardo-Daudt and Kennedy (2008) found Cd-induced endocrine disrupting effects in male and female rainbow trouts [*Oncorhynchus mykiss* (Walbaum, 1792)] exposed for 28 days, as well as hatching and developmental abnormities. In yet another study, Faucher et al. (2008) found that exposure to 0.5 μg Cd L^{-1} could alter the escape behaviour of *D. labrax*, presumably by affecting the lateral line system.

Flatfish (Teleostei: Pleuronectiformes) have long been regarded as important subjects for the biomonitoring of aquatic pollution due to their abundance, ecological and economical importance and, most importantly, to their benthic behaviour, since aquatic sediments, especially those of estuaries and other confined coastal waters, are major reservoirs of metals and other pollutants that may be released back to the water, thus increasing their bioavailability, when subjected, for instance, to disturbance and oxic–anoxic shifts (Eggleton and Thomas 2004). Species such as the European flounder [*Platichthys flesus* (L. 1758)], the English sole (*Parophrys vetulus* Girard, 1854) and the olive flounder [*Paralichthys olivaceus* (Temminck and Schlegel, 1846)], among others, take their part in many toxicological studies on feral animals, fish subjected to bioassays and even research in vitro (e.g. Myers et al. 1998; Li and Zhang 2001; Falciani et al. 2008 and also to the review by Cerdá et al. 2010). The Senegalese sole, *Solea senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae), is a well-represented flatfish in SE

Europe that occupies sandy–muddy floors of shallow coastal waters and estuaries (that act as important breeding and nursery grounds) where it preys on small invertebrates (Cabral 2000). It is a valuable species for fisheries and aquaculture and, in the past decade, taking advantage of its availability and ecological significance, the species has been employed in a growing number of studies in the field of environmental toxicology that survey histopathology and other effects and responses to toxicity caused, for example, by waterborne copper (Arellano et al. 1999), sediment-bound mixtures of contaminants (Costa et al. 2009, 2010, 2011) or the effects of intraperitoneally injected Cd on bioaccumulation and metallothionein induction (Kalman et al. 2010), to quote a few. Still, much research on the mechanisms and effects of toxicity is still missing to match other flatfish species, and no research is to be found regarding exposure to realistic concentrations of waterborne Cd.

The present work intends to perform a screening histopathological analysis on juvenile *S. senegalensis* exposed to waterborne Cd concentrations similar to those found in polluted environments. Specifically, it is intended to: (1) identify the most impacted organs; (2) to seek for dose- and time-effect relationships and (3) to provide a thorough and detailed description of histopathological lesions and alterations that may be surveyed as qualitative and semi-quantitative indicators of exposure in future works with Cd or other toxicants.

Methods and materials

Experimental procedure

Juvenile hatchery-brood and laboratory-reared Senegalese soles ($\approx$4 month old), all from the same cohort (46 $\pm$ 7 mm standard length; 1.2 $\pm$ 0.5 g total wet weight), were subjected to 28-day bioassays with three different nominal concentrations of dissolved Cd (obtained from a Merck $CdCl_2$ Tritisol solution): 0.5, 5 and 10 μg Cd L^{-1}, plus a control test. The assays were performed in duplicate in white polyvinyl tanks with blunt edges in which were placed twelve litres of Cd-spiked and control water plus six randomly selected fish per replica and test (no sediment was added). Photoperiod was set at 12:12 h light:dark and water temperature at $\approx$19 °C. The tanks were fitted with a recirculation apparatus and constant aeration (dissolved O_2 $\approx$ 60 % for all replicates). A partial water change (10 % total volume) followed by recontamination was done every 48 h in order to maintain bioassay conditions: salinity 30–31; ammonia <0.5 mg L^{-1}; pH 7.7 $\pm$ 0.2. Cadmium concentration in water was determined as quality control at the end of the bioassay procedure by differential pulse anodic stripping voltammetry with a hanging mercury drop electrode (DPASV-HMDE), using a Metrohm 694 VA stand and 693 VA processor (Costa and Costa 2008). Accordingly, Cd concentration for the control test was below the detection limit; 0.48 $\pm$ 0.17 μg L^{-1} for the 0.5 μg L^{-1} nominal concentration assay; 4.42 $\pm$ 1.07 μg L^{-1} for the 5 μg L^{-1} assay and 12.76 $\pm$ 1.64 μg L^{-1} for 10 μg L^{-1}. Fish sampling was performed after 14 (T_{14}) and 28 days (T_{28}) of exposure, with three animals being sampled per replica in order to obtain an $n = 6$ for each experimental condition. Animals were fed once a day with M2 grade commercial pellets (Sorgal, Portugal). Fish were euthanized by cervical sectioning immediately before processing for subsequent analyses.

Histopathological analyses

Whole fish were divided into head and trunk sections and fixed in Bouin–Hollande's solution (10 % v/v formalin; 7 % v/vacetic acid and picric acid added till saturation) for 36 h at room temperature. Samples were afterwards washed o/n in distilled water, dehydrated in a progressive series of ethanol (70, 95 and 100 % v/v) and embedded in paraffin (xylene was used for intermediate impregnation), also at room temperature. All sample batches were prepared following the same protocol to ensure tissue quality consistency. Sections (3–7 μm thick) were obtained with a Jung RM2035 model rotary microtome (Leica Microsystems) and mounted using a waterbath (set at 35 °C) on regular glass slides employing a standard albumin:glycerol adhesive and on Polysine-coated slides (Roth) for the TUNEL (TdT-mediated dUTP-X nick end labelling) assay. Sections were stained with Haematoxylin and Eosin (H&E) for general structural analyses, Periodic Acid-Shiff and Alcian Blue (PAS–AB) for histochemical detection of basic and acidic polysaccharides, respectively (Haematoxylin Gill no 3 was employed as counterstain), and by the TUNEL reaction using the In Situ Cell Death Detection Kit with fluorescein as fluorochrome (Roche Applied

Science). Other specific histochemical techniques, such as Perl's Prussian Blue (PB) for haemosiderin deposits with Nuclear Fast Red (NFR) as counterstain, Bronner's Sudan Black B (SB) for protein-bound lipids and the Acidine Orange fluorochrome (AO) for nucleic acids, were also employed. The TUNEL reaction was done according to the manufacturer instructions, all other methods are described in detail elsewhere (Martoja and Martoja 1967; Kiernan 2008). Slides stained with H&E, PAS–AB, AO and PB–NFR were mounted in DPX resin (BDH), all others with water. Observations were made in at least duplicate slides per animal and stain, from six to ten sections per slide, using a DMLB model microscope adapted for epifluorescence with an EL6000 light source for mercury short-arc reflector lamps, equipped with an I3 filter for fluorescein and a DFC model camera (all from Leica Microsystems). The histopathological screening was performed on multiple organs, with emphasis on the digestive and excretory systems, gills, skin and lymphoid organs. Since fish were young juveniles, gonad histopathology was not performed.

Statistics

Skin goblet (mucous secreting) cell measurements were compared between tests and sampling times as a quantitative indicator of response to exposure. Measurements (largest axial length) were obtained from goblet cells from the eyed side ($\approx$100 cells per individual). Pairwise comparisons between tests were also performed on fish size-related variables (standard length and total wet weight). The nonparametric Mann–Whitney *U* test and the Spearman *R* correlation statistic were employed upon failure to comply with parametric ANOVA assumptions, namely homocedascity (assessed by the Levene's test). The significance level α was set at 0.05 for all analyses. All statistics were performed with Statistica (Statsoft), after Zar (1998).

Results

No mortality occurred during the bioassays, and the sampled fish had no gross external lesions. No differences were found regarding either fish standard length or total wet weight between control animals and Cd-exposed soles, at any sampling time (Mann–Whitney $U, p > 0.05$). Internally, fish exposed to Cd, especially to 5 and 10 μg L^{-1}, at either sampling time, had a variable extension of hepatic inflammation. No other gross lesions and alterations were observed. No signs of parasites were detected internally or externally in any of the surveyed fish. The skeleton, skeletal muscle, heart, major blood vessels and central nervous system had no microscopic lesions. A series of blind reviews failed to demonstrate differences between the two experimental replicates; therefore, animals were grouped per treatment and sampling time for all subsequent analyses. Figure 1 illustrates the sections from which the histopathological screening was undertaken.

Digestive system

The liver was the only organ of the digestive system and annex glands to sustain any distinctive histological changes. No recognizable histopathological lesions and alterations could be found in oesophagus, midgut, hindgut and pancreatic acini in either control or Cd-exposed fish for any sampling time. Normal hepatic architecture consisted of a rosette arrangement of roughly polyhedric hepatocytes around a sinusoid, forming a hepatic cord-like structure. Normal hepatocytes possessed a translucent cytoplasm when stained by H&E where large reddish–pink granules can be observed in PAS-stained sections, which should indicate good glycogen storage (Fig. 2a). The general aspect of the livers of control fish collected at either sampling times is in good agreement with what has been previously described for normal juveniles of the species (e.g. Costa et al. 2009, 2011). Similarly to controls, the prevalence of lesions and alterations in fish exposed to the lowest Cd concentration (0.5 μg L^{-1}) was low but noticeable especially after 28 days of exposure (Fig. 2b). The most distinctive hepatic alteration was hyperaemia, likely caused by inflammation, evidenced by blood congestion in hepatic blood vessels causing sinusoidal swelling. Inflammation occurred in all tests after 28 days, however limited to small foci in control fish and fish exposed to the lowest concentration, whereas hyperaemia was very notorious in animal exposed to 5 and, especially, 10 μg cd L^{-1} even at T_{14}, reaching a diffuse pattern in soles exposed to the highest concentration for 28 days. Melanomacrophage intrusion occurred in fish exposed to Cd (especially 5 and

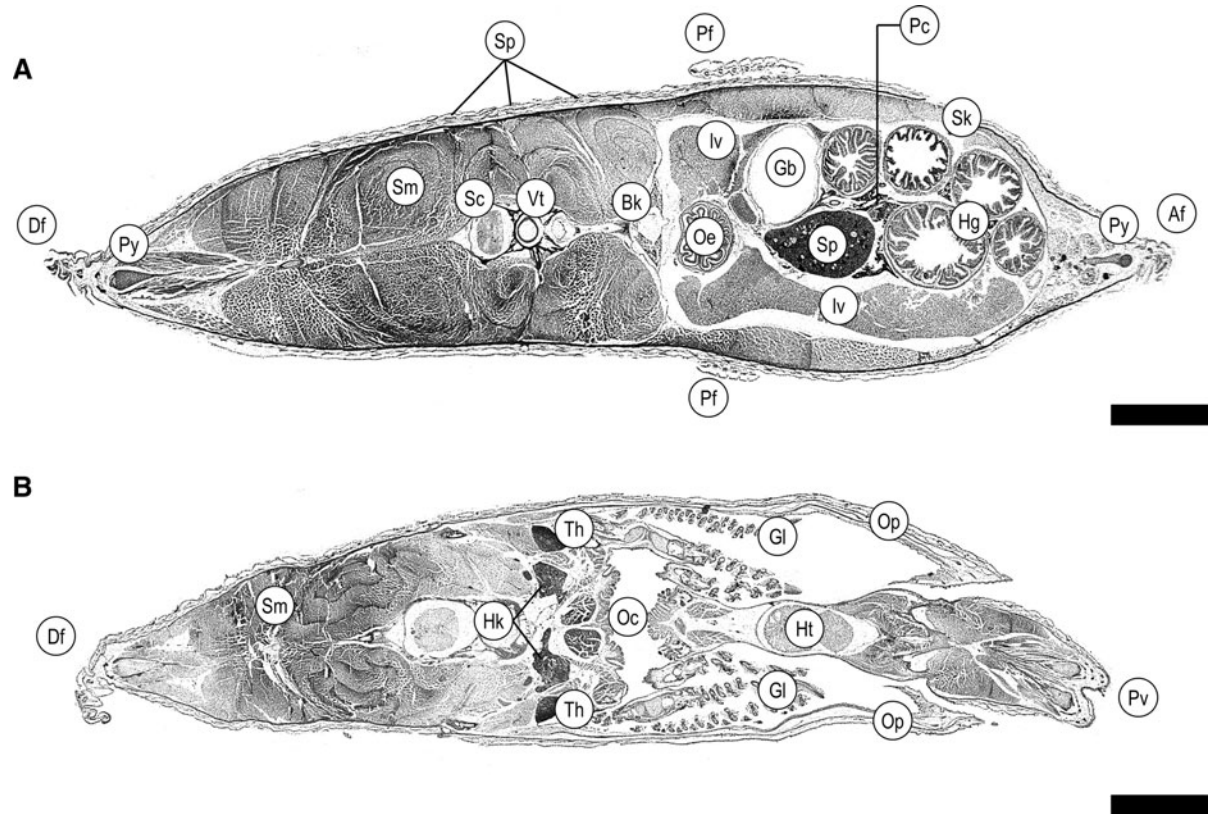

Fig. 1 Exemplificative histological sections of tested (28 days, 10 μg cd L^{-1}) *S. senegalensis* (ocular side upwards) showing gross internal anatomy (H&E). **a** Section through trunk. *Af* anal fin, *Bk* trunk kidney, *Df* dorsal fin, *Gb* gall bladder, *Hg* hindgut, *Lv* liver, *Mg* midgut, *Oe* oesophagus, *Pf* pectoral fins, *Py* pterygiophores (cartilaginous), *Sc* spinal cord, *Sk* skin with scale pouches (*Sp*), *Sm* skeletal muscle, *Sp* spleen, *Vt* vertebra. **b** Section through the opercular cavity. *Df* dorsal fin, *Gl* gill arches, *Hk* head kidney, *Ht* heart, *Oc* oral cavity, *Op* opercula, *Pv* base of pelvic fins, *Th* thymus. *Scale bar* 1 mm

10 μg L^{-1} and at later stages of exposure), occasionally forming dense centres near or at foci of apoptotic and necrotic hepatocytes (Fig. 2c). Hepatocyte apoptosis was infrequent except in animals exposed to the highest Cd concentrations and was identified by the presence of apoptotic bodies (typically compressed against blood vessels) that were found to be strongly PAS-, SB- and TUNEL-positive, which confirms the heterogeneous nature of the materials. Vacuolation, probably lipidic lipidosis, was a common hepatocellular alteration even in control and fish exposed to the lowest Cd concentration after 28 days of exposure. Lipid vacuoles appear as large, membrane-enclosed, empty structures inside cells, after lipid washing-off during sample processing with ethanol and xylene. Sudan Black staining failed to reveal any distinctive alterations to protein-bound lipids in any surveyed organ (not shown). Eosinophilic foci were present in animals exposed to 10 μg Cd L^{-1} collected at T_{28} (Fig. 2d). Glycogen depletion was variable, although a trend to intensify in fish exposed to the highest concentrations at a later stage of exposure was identified. Nuclear pleomorphisms (pyknosis or hypertrophy) were rare or absent in hepatocytes not undergoing cell death.

Trunk kidney

Trunk ("body") kidney alterations were highly variable and, with the exception of tubule and haematopoietic tissue apoptosis, almost exclusively limited to animals exposed to 5 and 10 μg Cd L^{-1}, although without a distinct dose- or time-dependent pattern. Control and 0.5 μg Cd L^{-1} fish possessed normal kidney structure throughout, with well-defined tubules lined with ciliated cuboidal epithelial cells (eosinophilic cytoplasm)

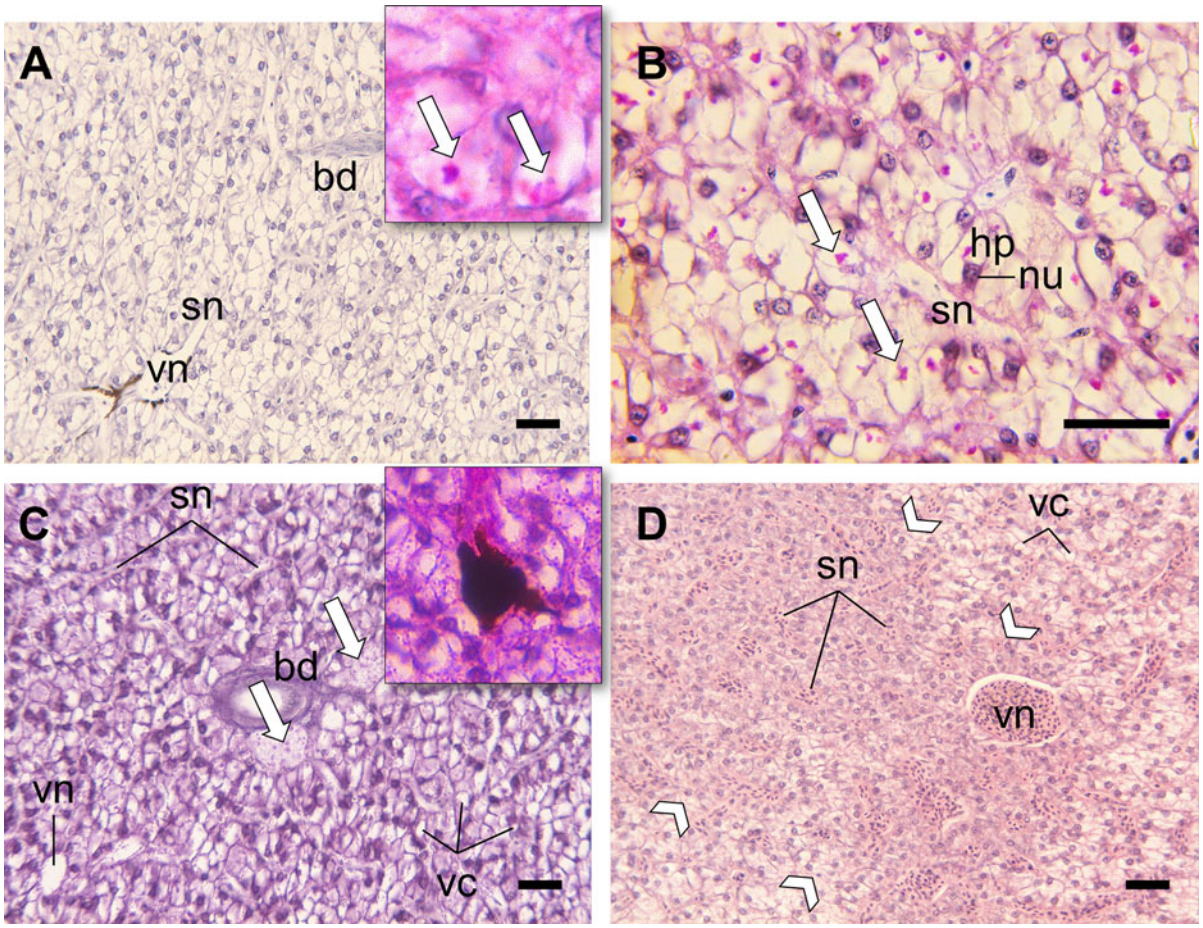

Fig. 2 Liver histopathology of tested individuals. **a** Normal hepatic parenchyma of a control fish (H&E) collected after 28 days of exposure, exhibiting regular hepatocytes, polyhedric in shape with well-defined nuclei and concentric nucleoli. Many sinusoids (*sn*) can be observed branching out of larger blood vessels, in the case, a hepatic venal branch (vn). Inset: hepatocytes around a sinusoid (forming a cord-like structure) from control fish after the same period (PAS–AB), exhibiting normal morphology and large PAS-positive cytoplasmatic granules, presumably glycogen (*arrows*). **b** Liver of a fish exposed to the lowest Cd concentration for 14 days (0.5 μg L^{-1}), depicting similar hepatic structure to those of controls, including large glycogen granules (*arrows*). *sn* sinusoid, *hp* hepatocyte, *nu* hepatocyte nucleus (PAS–AB). **c** Liver section of a sole exposed to 5 μg Cd L^{-1} for 14 days revealing multiple necrotic foci (*arrows*) adjacent to a bile duct (*bd*) (PAS–AB). Large PAS-positive granules are absent. *vc* vacuoles, probably lipidic, *sn* sinusoid, *vn* venule. *Inset* a dense melanomacrophage centre in the hepatic parenchyma of a fish from the same experimental treatment (PAS–AB). **d** Hepatic parenchyma of a fish exposed for 28 days to the highest Cd concentration (10 μg L^{-1}), revealing a large area of eosinophilic hepatocellular alteration (between a*rrowheads*); moderate fat vacuolation (*vc*) and diffuse inflammation throughout the section, inferred from hyperaemia in swollen sinusoids (*sn*) and venal branch (*vn*). H&E. *Scale bars* 25 μm

forming a distinct lumen (Fig. 3a). As expected in a marine teleost, Malpighian corpuscles were small and few in number, therefore insufficient for an objective histopathological analysis on glomeruli. Hyperaemia was a common alteration in animals exposed to the highest concentrations, indicating some degree of inflammation (Fig. 3b). Occasionally, blood-congested vessels were ruptured, causing localized haemorrhage (in fish exposed to 10 μg Cd L^{-1} only). Melanomacrophages frequently infiltrated towards apoptotic foci from nearby blood vessels, occasionally forming dense centres (Fig. 3c). Kidney tubule lesions consisted mostly of necrosis and vacuolation of epithelial cells (Fig. 3d). As for the liver, evident nuclear pleomorphisms were rare and did not account for any obvious trend.

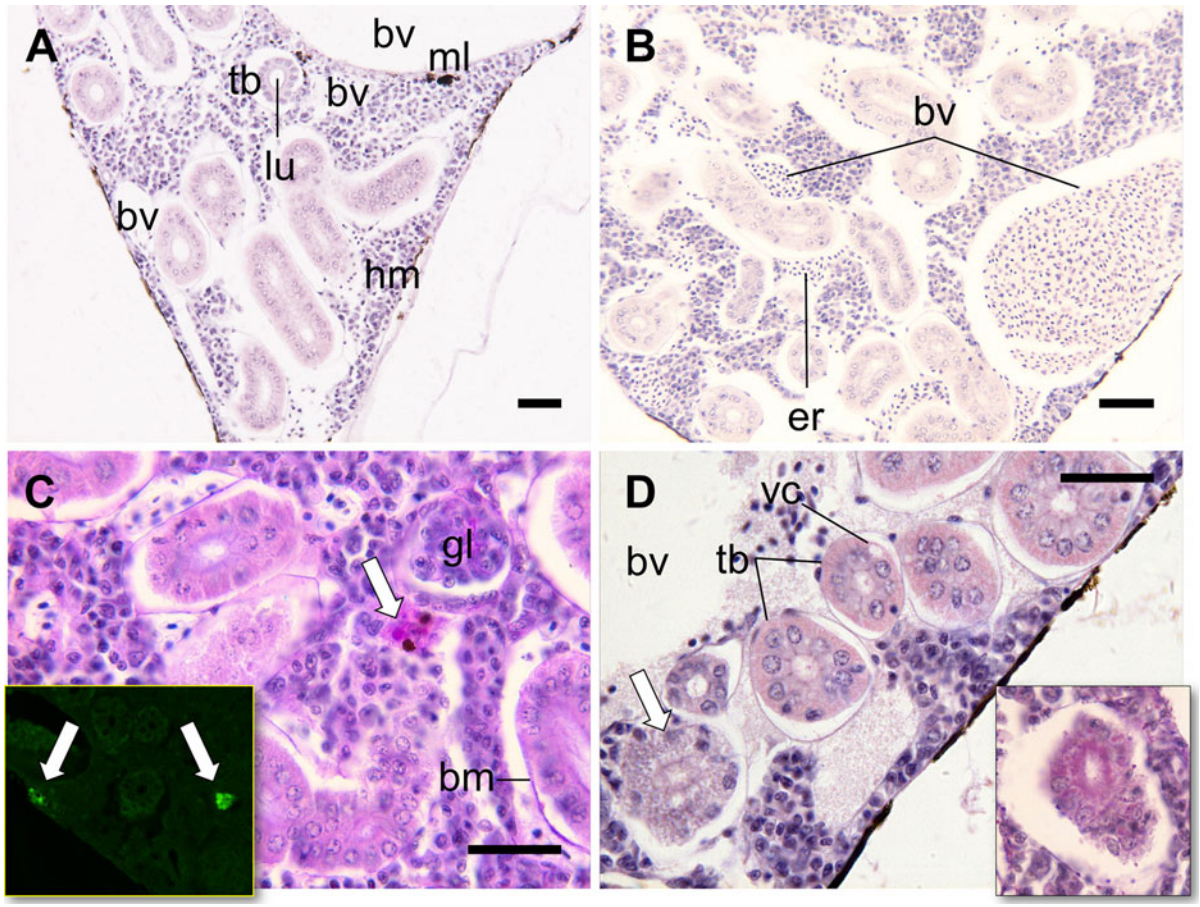

Fig. 3 Trunk kidney histopathology of tested soles. **a** Normal kidney from a control fish collected at the end of the experiment (T_{28}). Tubules (*tb*) are formed by regular cubic epithelial cells, have a well-defined lumen (*lu*) and are surrounded by haematopoietic tissue (*hm*) that also enclose many blood vessels (*bv*). Melanomacrophages (*ml*) can be observed on the haematopoietic tissue (H&E). **b** Inflammation in the kidney of a sole exposed to 10 μg Cd L^{-1} for 28 days, shown by hyperaemia in renal blood vessels (*bv*). Note focal erythrocytic intrusion into haematopoietic tissue (*er*). H&E. **c** Details of the trunk kidney of a fish exposed to 10 μg Cd L^{-1} for 14 days (PAS–AB) exhibiting foci of apoptotic tubules, generally accompanied by melanomacrophages phagocytising the apoptotic bodies, usually strongly PAS-positive (*arrow*). The basal membrane of renal epithelia stains strongly with Alcian Blue (*bm*). *gl* indicates a normal glomerulus. *Inset* section of a fish exposed to 10 μg Cd L^{-1} for 14 days and stained through the TUNEL reaction (and viewed under epifluorescence) confirming the presence of apoptotic cells (*arrows*) in haematopoietic tissue (*left*) and tubules (*right*). **d** kidney of a sole exposed to 10 μg Cd L^{-1} for 28 days, revealing an early-stage necrotic tubule (*arrow*) between tubules (*tb*) where the only noticeable alterations were intraplasmatic vacuoles (*vc*). *bv* blood vessel. H&E. *Inset* a more advanced stage of tubule necrosis in a fish exposed to 5 μg Cd L^{-1} for 14 days (PAS–AB). *Scale bars* 25 μm

Immune system-related organs

Comparative to all other treatments, the spleen of soles exposed to 10 μg Cd L^{-1} for both 14 and 28 days presented highest damage, presented as multiple foci of necrotic haematopoietic tissue, reaching the level of diffuse dissemination (Fig. 4). Melanomacrophages formed centres near or at these foci. Large haemosiderin granules revealed by the Prussian Blue method were often found on necrotic foci targeted by melanomacrophages; however, it is likely that fixation with an acetic acid-containing solution such as Bouin–Hollande's facilitated the release of iron prior to staining, and therefore, such observations may be underestimated. Splenic lesions in all other tests were low or absent. The thymus, an essentially lymphopoietic organ located in the anteriolateral portion of the branchial cavity was also screened for alterations, but

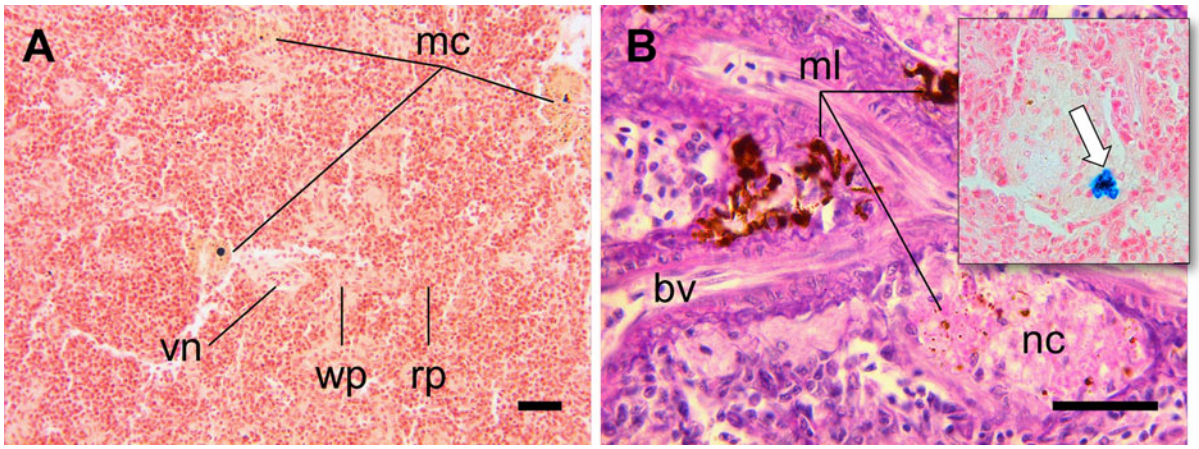

Fig. 4 Example micrographs of splenic tissue of soles exposed to the highest concentrations of Cd (10 μg L^{-1}). **a** Moderately damaged spleen of an animal exposed for 14 days with melanomacrophage centres revealed by the aggregation of *dark brown* (melanin-like) or *yellowish* (lipofuscin) vesicles. *rp* red pulp (composed mostly of reticulocytes and erythrocytes), *vn* venule, *wp* white pulp (mostly lymphoid cells). H&E. **b** Details of the spleen of a fish exposed for 28 days with multiple necrotic foci (*nc*). Melanomacrophages (*ml*) are observed to creep out of blood vessels (*bv*), in this case a branching splenic arteriole, onto a necrotic area. PAS–AB. *Inset* large haemosiderin granules (*arrow*) in a necrotic focus. PB&NFR. *Scale bars* 25 μm

no discernible lesions occurred in fish subjected to any treatment. Rodlet cells, nowadays linked to immunity functions in teleosts, including flatfish (e.g. Vigliano et al. 2009), could only rarely be positively identified in any of the surveyed epithelia (especially skin, gills and gut).

Gills and skin

No lesions were present in the skin of fish subjected to any test, including to lateral line system sensory organs (neuromasts) and chemoreceptors (cutaneous taste buds). Epidermis was constituted by an outermost layer of stratified epithelium and a basement layer of larger, undifferentiated, cells attached to the epithelium basal membrane. Dermis was formed mostly by connective tissue and includes the scale pockets. Skin structure was consistent with normal teleost integument architecture (refer, e.g., to Hawkes 1974). Epidermal goblet (mucous secreting) cells were present in larger numbers in the ocular side of the fish, similar to what has been described for other flatfish species (Yamamoto et al. 2011). Goblet cells had a vesicular cytoplasm and were observed to be mostly AB-positive, although PAS-positive cells or cells stained by both could also be observed. The distribution of goblet cells through the skin of the ocular side around the lateral line, where measurements were performed, was uneven and did not vary between experimental treatments (averaging 25–45 cells mm^{-1} skin section). However, there were differences on the average size of goblet cells, with soles exposed to 5 μg Cd L^{-1} presenting a significantly increased goblet cell size compared to controls (Mann–Whitney U, $p < 0.05$), after 28 days of exposure, with no significant differences being detected to other treatments (Fig. 5a, b). However, at T_{14}, the only significant difference observed concerns the larger size of goblet cells of animals exposed to 5 μg L^{-1} comparative to 0.5 μg L^{-1} (Mann–Whitney $U, p < 0.05$). Fish exposed to the lowest Cd concentration (0.5 μg L^{-1}), the only treatment where a significant increase in goblet cell size from T_{14} to T_{28} occurred. A positive correlation was obtained between average goblet cell size and water Cd concentrations for T_{14} only (Spearman $R = 0.60$, $p < 0.05$).

Gill damage was variable, however more pronounced in fish exposed to the highest Cd concentrations but without a clear relationship with either time of exposure and Cd concentration in water. Still, in general, the prevalence of gill lesions was low and mostly localized, with the exception of the marked chloride cell hypertrophy present in some of the animals exposed to the highest Cd concentrations, at either sampling time (Fig. 6a). Hypertrophied chloride cells presented a similar aspect to that observed in

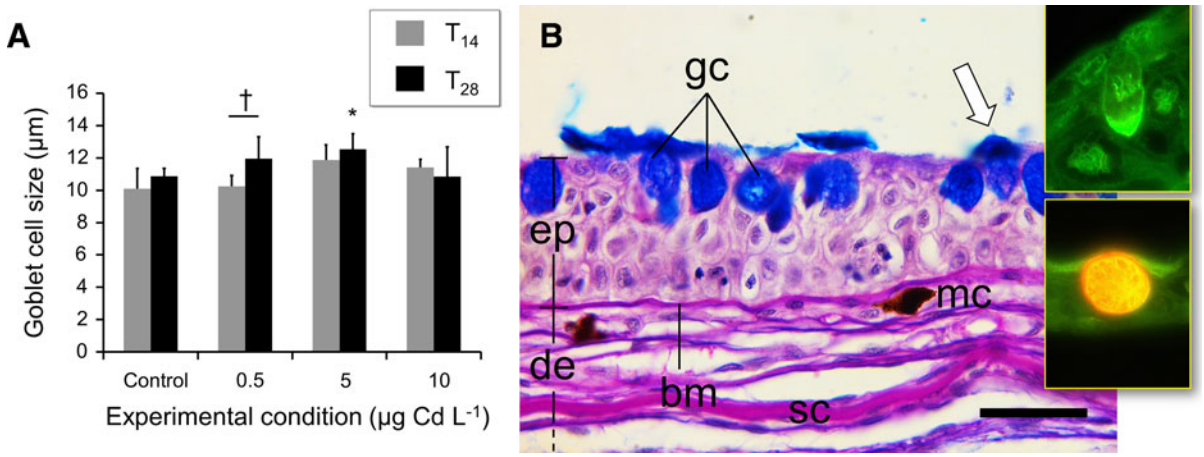

Fig. 5 Histopathological evaluation of the skin of tested animals. **a** Goblet (mucous) cell size variation for all tests, comparing both sampling times. *Asterisk* indicates significant differences to control at respective sampling time; (†) means significant differences between animals collected after 14 (T_{14}) and 28 (T_{28}) days of exposure. Statistics were obtained with the Mann–Whitney *U* test ($p < 0.05$). *Error bars* mean standard deviation. **b** Skin section of a sole exposed to 10 µg Cd L^{-1} for 28 days (PAS–AB). Comparatively to control animals, even in fish exposed to the highest nominal concentrations, the structure of the integument remained essentially unaltered, with the exception of an engorgement of PAS- or AB-positive (most frequently) goblet cells (*gc*) that protrude mucous in a typical dome shape (*arrow*). *bm* basal membrane of the epidermis, *de* dermis, *ep* epidermis, *mc* pigment cell (melanocyte). *Scale bar* 25 µm. *Inset* secretory epidermal cells stained with AO, detailing probably a rodlet cell (*above*) with rod-shaped strongly AO-positive structures inside (thus likely to contain nucleic acids) and an orthochromatic (*reddish-orange*) goblet cell contrasting with remaining *yellowish-green* (AO metachromatic) adjacent cells (*below*). The *reddish colour* is given by the mucous vesicles' membranes and not by the mucous itself. (Color figure online)

other studies and are apparently caused by intraplasmatic fluid retention, rather than vacuolation, whereas normal chloride cells are mitochondria-rich and are strongly stained by H&E (Arellano et al. 1999; Costa et al. 2010). Although infrequent, deposits of inorganic, crystalline-resembling deposits (likely metallic) were present inside epithelial cells in the gills of the most affected fish. Other lesions and alterations to gills included low-moderate hyperplasia of interlamellar space epithelia, detachment of basal membrane of squamous epithelia of lamellae (leading to epithelial lifting), desquamation of lamellar epithelia and lamellar deformation (Fig. 6b). No circulation- or inflammation-related disorders occurred in fish subjected to any tests as well as any apparent changes to gill goblet and normal chloride cell number and size.

Global histopathological evaluation

Besides changes to skin goblet cell size, twenty-eight clear histopathological lesions and alterations could be pinpointed in four distinct organs, kidney, liver, spleen and gills. On aggregate, the liver and trunk kidney of fish exposed to Cd accumulated more lesions and alterations, contributing with eight histopathological each, followed by the gills with five and spleen with two. The most disseminated alterations occurred in fish exposed to the highest Cd concentrations and related to inflammation in the livers and kidneys; chloride cell hypertrophy in the gills and splenic necrosis. Liver inflammation was also the most evident alteration in fish exposed to 0.5 µg Cd L^{-1}. Table 1 summarizes the results from the qualitative histopathological approach.

Discussion

Although it is generally considered that Cd is primarily nephrotoxic and then hepatotoxic as a consequence of chronic exposure (refer, for instance, to Nordberg 2009 and Nawrot et al. 2010), it was the liver that, in the present study, sustained histological lesions and alterations more clearly relatable to Cd concentration and time of exposure. The alterations correspond especially to inflammation-related responses (as hyperaemia and macrophage intrusion). Direct lesions such as necrosis had a low prevalence, although a trend to

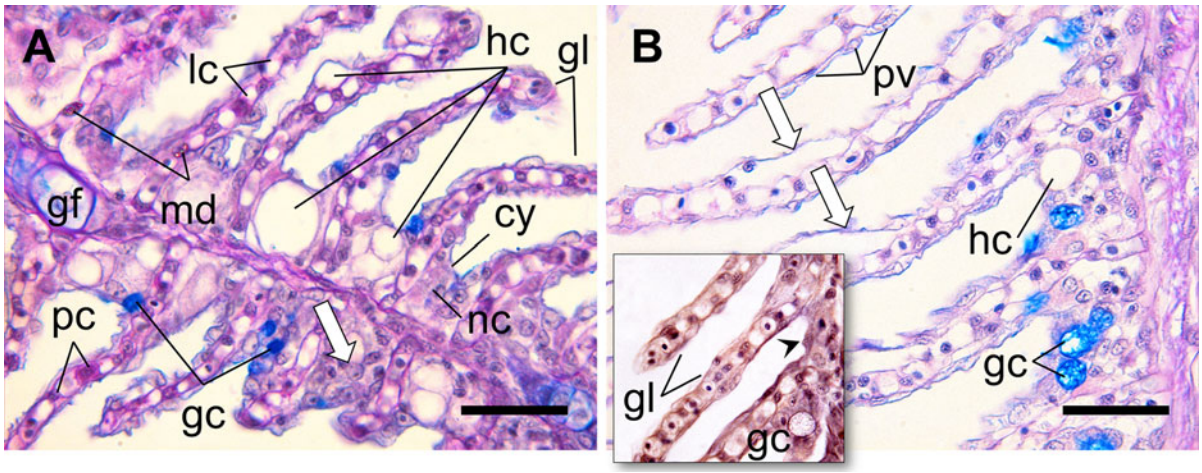

Fig. 6 Gills of fish exposed to the highest Cd concentrations (PAS–AB). *Scale bars* 25 μm. **a** Details of a gill filament (*gf*) of a sole exposed 5 μg Cd L^{-1} for just 14 days, revealing diffuse hypertrophy of chloride cells (*hc*). Chloride cells gain a "vacuolated" aspect due to liquid retention and increased size. Normal chloride cells (*nc*) are easily distinguishable by their smaller size, dense cytoplasm and conspicuous crypt (*cy*). Moderate hyperplasia of epithelial cells can also be observed between two bent lamellae that upon sectioning resemble shorter than the remaining (*arrow*), as well as a few intracellular metal deposits (*md*). *gc* goblet cells (AB-positive), *gl* gill lamellae, *lc* lamellar capillaries, *pc* pillar cells. The goblet cells' apparent reduced size in this micrograph is incidental (due to the plane of sectioning). No apparent change in goblet cell morphology was observed in any test. **b** Lifting of the squamous epithelium basal membrane (*arrows*) in the gill of a fish exposed to 10 μg cd L^{-1} for 28 days. Compare to normal arrangement of pavement cells (*pv*). A hypertrophied chloride (*hc*) cell may also be observed in the interlamellar space. *gc* goblet cells (AB-positive). *Inset* normal lamellae (*gl*) and interlamellar space (*arrowhead*) of a control fish collected after 14 days. *gc* goblet cell (H&E)

increase with time and concentration of exposure was identified, showing that exposure to low concentrations of this toxic metal may in fact elicit chronic hepatic damage to juvenile *S. senegalensis*. The occurrence of eosinophilic foci in the livers animals exposed to 10 μg Cd L^{-1} may confirm this premise, since some authors regard this alteration as potentially related to a pre-neoplastic condition or at least common in livers undergoing pre-neoplastic changes (Vogelbein et al. 1990; Vethaak and Wester 1996). However, no definitive data still exist linking this specific hepatocellular alteration to tumourigenesis in fish. It should also be noticed that, when comparing to controls, liver inflammation also occurred in fish exposed to the lowest Cd concentration (0.5 μg L^{-1}); although at a later stage, when analyses of other organs failed to reveal distinguishable alterations as a consequence of exposure to the metal. Overall, according to the severity and degree of dissemination of the histopathological changes observed and to the circumstances of assessment, the organs where lesions were found could be ranked as (from the most to the least affected): liver > kidney > spleen > gills > skin. It may thus be inferred that, considering the low concentrations tested and the assays' duration, this ranking is likely to reflect the organs' specific sensitivity to Cd in young, metal-naïve, soles.

Previous research often shows inconsistent findings regarding differential toxicity of Cd to the liver and kidney (and well as on bioaccumulation), revealing Cd transport between the organs as a function of its binding to metallothioneins (MTs) since MT-bound Cd (CdMT) is chiefly formed in the liver, then transported to the kidneys, being inorganic Cd (as $CdCl_2$) more toxic to the liver than to the kidney, since CdMT is easily excreted by the glomeruli and afterwards reabsorbed by the tubules, which releases Cd into the epithelial cells (see Groten et al. 1991; Dorian et al. 1995 and also Nordberg 2004 for a review). Time of exposure is a critical factor since it is likely that longer bioassays would have enhanced Cd bioaccumulation, thus rendering more notorious the adverse effects hereby scored. In addition, there is little or no information on these processes in marine fish, which have few, reduced, glomeruli and different renal architecture from the mammalian models that benefit from most of the research dedicated to the subject.

Table 1 Minimum–maximum range of the most important histopathological lesions and alterations in tested Senegalese soles

Organ	Nominal Cd exposure (μg Cd L^{-1})							
	Control		0.5		5		10	
	T_{14}	T_{28}	T_{14}	T_{28}	T_{14}	T_{28}	T_{14}	T_{28}
Trunk kidney								
Tubules								
Apoptosis	0–1	0–1	0–1	0–1	0–2	1–1	1–1	1–1
Necrosis	n.o.	n.o.	n.o.	n.o.	0–1	0–1	0–1	0–1
Vacuolation	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0–2
Haematopoietic tissue								
Apoptosis	0–1	0–1	0–1	0–1	1–2	1–2	1–2	1–2
Necrosis	n.o.	n.o.	n.o.	n.o.	1–2	1–2	0–1	0–2
Circulation/inflammation								
Haemorrhage	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0–2
Hyperaemia	n.o.	n.o.	n.o.	n.o.	1–2	1–2	1–2	2–3
Melanomacrophage intrusion	0–1	0–1	0–1	0–1	1–2	1–2	1–1	1–2
Liver								
Hepatocellular alterations								
Apoptosis	n.o.	n.o.	n.o.	0–1	0–2	0–2	0–2	0–2
Eosinophilic foci	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	1–2
Glycogen depletion	n.o.	0–1	n.o.	n.o.	1–1	1–2	1–2	2–2
Lipidosis	n.o.	0–2	n.o.	0–1	1–2	2–2	1–2	2–2
Necrosis	n.o.	n.o.	n.o.	0–1	1–2	1–2	1–2	1–2
Circulation/inflammation								
Exudate	n.o.	n.o.	n.o.	0–1	1–2	1–3	2–3	2–3
Hyperaemia	n.o.	0–1	0–1	1–2	1–2	1–3	2–3	2–3
Melanomacrophage intrusion	n.o.	n.o.	n.o.	n.o.	0–1	1–2	0–1	1–2
Spleen								
Haematopoietic tissue								
Melanomacrophage intrusion	n.o.	n.o.	0–1	0–1	0–1	0–1	1–2	1–3
Necrosis	n.o.	n.o.	0–1	0–1	0–1	1–1	1–2	2–3
Gills								
Morphology								
Lamellar deformation	n.o.	n.o.	n.o.	n.o.	0–1	0–1	0–1	0–1
Epithelia								
Chloride cell hypertrophy	n.o.	n.o.	n.o.	n.o.	0–3	1–3	0–3	1–3
Desquamation	n.o.	n.o.	n.o.	n.o.	0–2	0–2	0–2	0–2
Detachment of basal membrane	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0–2
Hyperplasia	n.o.	n.o.	n.o.	n.o.	0–1	0–1	0–1	0–1

0, absent; 1, infrequent; 2, frequent; 3, diffuse; *n.o.* not observed in any fish from the respective test and sampling time

Cadmium is long known to disrupt hepatic carbohydrate metabolism, leading to a decrease in glycogen storage and increased plasma glucose, as observed for instance by Soengas et al. (1996) in Atlantic salmon (*Salmo salar* L.) exposed to 10 and 100 μg Cd L^{-1}, which is in accordance with the present work, where a decrease of glycogen storage in fish exposed to highest Cd concentrations occurred; however, contradicting other reports, such as Thophon et al. (2003), who observed increased glycogen in the livers of *Lates*

calcarifer (Bloch, 1790) exposed to 800 μg Cd L^{-1} for 90 days, presumably by a decrease of carbohydrate catalytic enzymes. Still, the same authors also report an increase in liver lipid accumulation (in the form of lipidosis), as observed in the current study. Even though there is a distinct order of magnitude regarding Cd concentrations between this latest report and the present work, it may be inferred that, in either situation, exposure to Cd disrupted carbohydrate metabolism. However, caution is needed when interpreting lipidosis since this alteration has many causes and may occur in control fish, probably deriving from feeding during the bioassays, as observed from previous studies also (Costa et al. 2009, 2011).

Cadmium-induced renal tubule apoptosis is thought to derive from the indirect formation of ROS (reactive oxygen species) by the metal (Cd itself is not a Fenton metal and therefore cannot generate oxidative radicals on its own) and by zinc (Zn) displacement from nuclear zinc finger proteins involved in the detection and repair of damaged DNA (see Hamada et al. 1997, for a review). This information aids explaining the modest increase in the evidence for apoptosis in the kidneys and livers of fish exposed to Cd comparatively to controls. Apoptosis is a programmed cell death (PCD) pathway in animals that essentially "dismantles" cells damaged beyond repair to avoid heavy inflammation caused by necrosis, dissemination of toxic cellular debris and propagation of cells containing mutated (damaged) DNA (Häcker 2000). Although a trend to increase at higher exposures, no clear time effect was observed on apoptosis, which revealed, in addition, similar prevalence as necrosis in both kidney and liver.

Despite Cd being known as a cause of immunosuppression and anaemia (e.g. Horiguchi et al. 2011), little is known about the mechanisms of Cd toxicity to the spleen. Lemaire-Gony et al. (1995) reported that *D. labrax* exposed to a single concentration of 40 μg Cd L^{-1} caused a reduction in the phagocytic ability of spleen macrophages. Giari et al. (2007), in a study with the same species, also found evidence for time- and water Cd concentration-dependent increased number of melanomacrophage aggregates in multiple organs, including the spleen; however, the relation with splenic tissue damage was not studied. Still, it is known that haemosiderin deposits are formed inside melanomacrophages in haematopoietic organs when degradation of erythrocytes and haematopoietic tissue occurs (see Agius and Roberts 2003, for a review). In the present study, exposure to the highest Cd concentration (10 μg L^{-1}) could elicit diffuse splenic necrosis. Although at this point, it is not possible to clearly state whether the iron deposits observed are a result of increased haemolysis, necrosis or a consequence of an unbalancing of iron metabolism, it may be inferred that exposure to low concentrations of Cd may cause adverse effects on *S. senegalensis* blood and immune system by affecting the spleen, which is in accordance with the results obtained by Johansson-Sjöbeck and Larsson (1978) with flounders exposed to 5–500 μg Cd L^{-1}. Brumbaugh et al. (2005), for instance, found Cd in the blood of feral freshwater fish collected from metal-contaminated areas to be well correlated to bioaccumulation in other organs, especially the liver, which reinforces that the toxic effects of the metal on peripheral blood, and therefore to haematopoietic organs, should not be disregarded. Still, a more prolonged exposure would be needed to see whether damage could occur in another haematopoietic organ, the thymus, and to draw more solid conclusions from dose- and time of exposure-related effects.

Skin and, especially, gills are the likely primary entry point of waterborne Cd, which is in accordance with the absence of lesions in the digestive tract, well-known to be impacted in fish exposed to the metal via food items (e.g. Berntssen et al. 2001), even though changes to intestine epithelia have also been found in fish exposed to high concentrations of waterborne Cd (for instance Giari et al. 2007; Isani et al. 2009). Although no obvious skin lesions were found in the present study, histopathological changes to fish integument exposed to waterborne toxicants, including Cd, have been described by other authors, however, details on exposures to low concentrations are scarce or even absent. Iger et al. (1994) described a series of both dermic and epidermal alterations (from necrotic pavement epithelium to fibrous tissue alterations) in *Cyprinus carpio* L., without an obvious dose-effect relationship at earlier stages of exposure—in a study where the lowest concentration tested was 22 μg L^{-1}. Literature on the comparative effects of Cd and other toxicants to gills and skin are essentially absent. Still, Handy (1992) described reduced Cd bioaccumulation in skin compared to liver, kidney and especially gills, where highest Cd concentrations were observed, in trouts exposed to 0.1 and 0.2 mg L^{-1}, but the gills

were found to be the most efficient organ detoxifying the metal. Thophon et al. (*op. cit.*) also found the gills to be less affected, as in the present work, than liver and kidneys of *L. calcarifer* exposed to sub-lethal Cd concentrations. The results are also in accordance with the previous studies that showed far less cumulative damage (in severity and dissemination) to gill epithelia in juvenile *S. senegalensis* exposed to complex, toxic mixtures of substances that elicited pronounced damage to other organs, namely kidney and liver (Costa et al. 2009, 2010). The current findings showed that the gills were more prone to acquire histopathological lesions than skin as a result of exposure to low Cd concentrations, which should indicate differential response and defence mechanisms between the two organs. Regarding these, changes to skin goblet cell (size, distribution and chemical composition) as a consequence of external insult have already been reported. A reduction in skin goblet cell size in fish, including flatfish, has already been found to occur as a result of different factors, from bacterial infections (Yamamoto et al. 2011) to exposure to sediment-bound contaminants (Mézin and Hale 2000). Some authors have discussed that a reduction in goblet cell size should not mean mucous production impairment but rather increased secretion/production ratios (refer to Mézin and Hale 2000). Goblet cell reduction also occurred in gill epithelia of *S. senegalensis* exposed to contaminated sediments without, however, evidence for increased mucous secretion (Costa et al. 2009). Nevertheless, this subject is yet little studied regarding exposure to contaminants. The present study revealed a moderate increase of goblet cell size, especially in fish exposed to the intermediate Cd concentration (5 μg L^{-1}), which may reflect a defence mechanism against waterborne Cd challenge in order to protect the integument. Conversely, a defensive increase in skin mucous secretion may have been compromised in fish exposed to 10 μg Cd L^{-1} as a direct or indirect consequence of toxicity itself. Maunder et al. (2011) found increasing Cd concentrations in the skin mucous of the freshwater cichlid *Symphysodon* sp. exposed to water or dietary Cd, which adds the relevance of goblet cells in Cd detoxification. Overall, it is likely that skin, as for gill epithelia, for being the first line of contact against any waterborne toxicant, increased mucous production as a defence mechanism, which may be inferred from the positive correlation between goblet cell size and nominal Cd concentration in T_{14}-collected animals. However, the absence of a significant correlation at T_{28} should indicate that the increase in mucous production is asymptotic, therefore compromising dose-effect determinations at more prolonged exposures, although it may be an indicator of exposure to a chemical stressor as Cd.

The gill epithelium is the main apical entry surface for waterborne contaminants in fish. Although variable, the present study found a trend to increase changes to gill epithelia with increasing concentrations of Cd, with especially regard to chloride cell hypertrophy by intraplasmatic fluid retention, an alteration that is likely to compromise ion excretion, a crucial physiological process in marine fish to maintain internal osmotic balance (refer, e.g., to Karnaky et al. 1976a, b). This specific alteration (not found in controls and animals exposed to the lowest Cd concentration) has been recorded in *S. senegalensis* exposed to mixtures of organic and inorganic toxicants (Costa et al. 2010) and copper (Arellano et al. 1999). However, Alvarado et al. (2006) failed to find this specific alteration in another flatfish (*Scophthalmus maximus* L., 1758) exposed to 1 and 10 mg L^{-1} of Cd (or other metals), even though chloride cell hyperplasia was recurrent and regarded as a defence mechanism to excrete excessive metal that was linked to MT induction and metal deposits. As for other issues, information on exposure to lower Cd concentrations is scarce. Still, differential sensitivity between species should not be excluded, likely linked to distinct mechanisms and thresholds of response. It is possible that at least some of soles exposed to the highest Cd concentrations had osmotic balance considerably impaired; as well as Cd elimination by chloride cells, leading to increased gill damage and higher availability of the metal to enter the blood stream and affecting other organs.

The present study showed that exposure to concentrations as low as 0.5 μg Cd L^{-1}, that is, within the range of realistic environmental contamination, on a metal-naïve juvenile marine teleost can trigger a broad range of histopathological changes to multiple organs, in a short-medium time range. Furthermore, flatfish, and soleids in particular, inhabit estuaries and other confined coastal waterbodies frequently impacted by hazardous metals such as Cd, which renders *S. senegalensis* juveniles susceptible to sustain Cd-induced injury even at low levels of contamination. The histopathological changes observed occur in a progressive time- and

concentration-related series: organs that form the organism-water boundary (gills and skin) were the least affected, followed by the spleen, trunk kidney and finally the liver as the most affected. The differences in the severity and degree of dissemination of histopathological lesions and alterations are a probable function of tissue-specie defences and sensitivity and, importantly, the mechanisms of Cd translocation within the organism, from water to gills, then to blood which conveys the metal to other organs, especially the liver, followed by the kidney and spleen, which is likely impacted through the recycling of Cd-affected blood cells. By its turn, the kidney is impacted especially by the impairment of tubular active transport, as in gill chloride cells (e.g. van Kerkhove et al. 2010). The results also indicate that, although no specific biomarkers of exposure to Cd could be distinctively pin-pointed, the analyses of multiple organs may reveal a histopathological pattern indicative of exposure to low concentrations of a toxic metal that include identifiable traits such as focal cell death, inflammation, changes that reveal alterations to carbohydrate metabolism (as glycogen depletion and lipidosis), osmotic balance impairment (inferred from gill chloride cell hypertrophy and kidney tubule lesions) and skin goblet cell engorgement revealing increased mucous production as a response/defence mechanism. Overall, the histopathological lesions and alterations observed are consistent with chronic disease and may be surveyed in future research for qualitative or semi-quantitative assessment (as histopathological indices) of metal-induced injury either in the laboratory or, most importantly, in field studies, where low background levels of Cd contamination apply.

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