Ecotoxicology (2012) 21:1194–1207
DOI 10.1007/s10646-012-0874-7

Hepatic proteome changes in *Solea senegalensis* exposed to contaminated estuarine sediments: a laboratory and in situ survey

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Accepted: 11 February 2012 / Published online: 24 February 2012
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Abstract Assessing toxicity of contaminated estuarine sediments poses a challenge to ecotoxicologists due to the complex geochemical nature of sediments and to the combination of multiple classes of toxicants. Juvenile Senegalese soles were exposed for 14 days in the laboratory and in situ (field) to sediments from three sites (a reference plus two contaminated) of a Portuguese estuary. Sediment characterization confirmed the combination of metals, polycyclic aromatic hydrocarbons and organochlorines in the two contaminated sediments. Changes in liver cytosolic protein regulation patterns were determined by a combination of two-dimensional electrophoresis with de novo sequencing by tandem mass spectrometry. From the forty-one cytosolic proteins found to be deregulated, nineteen were able to be identified, taking part in multiple cellular processes such as anti-oxidative defence, energy production, proteolysis and contaminant catabolism (especially oxidoreductase enzymes). Besides a clear distinction between animals exposed to the reference and contaminated sediments, differences were also observed between laboratory- and in situ-tested fish. Soles exposed in the laboratory to the contaminated sediments failed to induce, or even markedly down-regulated, many proteins, with the exception of a peroxiredoxin (an anti-oxidant enzyme) and a few others, when compared to reference fish. In situ exposure to the contaminated sediments revealed significant up-regulation of basal metabolism-related enzymes, comparatively to the reference condition. Down-regulation of basal metabolism enzymes, related to energy production and gene transcription, in fish exposed in the laboratory to the contaminated sediments, may be linked to sediment-bound contaminants and likely compromised the organisms' ability to deploy adequate responses against insult.

Keywords Proteomics · Flatfish · Bioassays · Sediment contamination · Sado Estuary

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Introduction

Assessing the impact of sediment-bound contamination to organisms in estuaries is an acknowledged challenge due to the complex geochemistry of estuarine sediments, to the strong anthropogenic pressure put onto estuaries and to the particular physico-chemical nature of transition water bodies. In addition, the deposition and release of contaminants trapped in estuarine sediments is complex and depends on multiple factors such as substance speciation, sediment organic carbon load, grain size, disturbance and

shifts in the oxic/anoxic status, just to account for some important abiotic factors (Atkinson et al. 2007). The combination of all these parameters with the most probable presence of multiple classes of contaminants constitutes a substantial confounding factor when assessing either the potential impact of sediment-bound contamination to organisms. In fact, nowadays there is a strive to find biomarkers for environmental contaminant mixtures (Monserrat et al. 2007), since such mixtures have been found to produce results that may contradict expected "classic" biomarker responses (e.g., Triebskorn et al. 1997; Mouneyrac et al. 2002; Costa et al. 2009, 2010a). Bringing the biomarker approach to a new, state-of-the-art, molecular level, high throughput "omics" techniques began over the past decade to be employed in eco- and environmental toxicology. Such research has, in most cases, intended to survey patterns of response, novel potential biomarkers and to contribute to the understanding of the cellular processes that underlie chemical insult, taking advantage of the screening-based approach that these methods rely on (López-Barea and Gómez-Ariza 2006; Monsinjon and Knigge 2007), and has been successfully extended to piscine species (see Forné et al. 2010, for a recent review on fish proteomics).

Either for the purpose of ecological risk assessment (ERA) or for more mechanistic approaches to toxicity assessment, bioassays are the backbone of much research with aquatic organisms. Still, although some authors already reported differences between laboratory and field assays with fish for the purpose of biomonitoring natural sediments (for instance, Vethaak et al. 1996; Hatch and Burton 1999), little research exists comparing the two types of bioassays directly and fewer or none reporting on "omics". Regardless of the methodology to determine the effects and responses to toxicity in organisms, such comparison has been determined of relevance since field (in situ) assays are affected by environmental variables other than contamination; laboratory assays, on the other hand, are rarely ecologically realistic and tend to be conservative (Chapman 2007).

Among fish species that have been employed in environmental toxicology studies, flatfishes (Teleostei: Pleuronectiformes) have been receiving growing attention for the environmental monitoring of potentially contaminated sediments due to their benthic disposition. Complementarily, studies focusing on more mechanistic approaches using these animals as models with the application of "omics" are springing out (Cerdà et al. 2010). Species like the flounder (*Platichthys flesus*) have been responsible for a large number of publications in ecotoxicology and have recently met their SW European counterpart, the soleid *Solea senegalensis*. The Senegalese sole, *Solea senegalensis* Kaup, 1858, is a flatfish of important value for fisheries and, especially, for aquaculture in Southern Europe. In its natural habitat, the species inhabits muddy or sandy floors (where it feeds on small invertebrates) of coastal areas, especially estuaries, since they constitute important breeding and nursing grounds (Cabral and Costa 1999; Cabral 2000). Much research has been performed on this species in the areas of aquaculture, marine ecology and ecotoxicology. "Omics" (proteomics and transcriptomics) techniques have, inclusively, been performed on Senegalese soles, although more for aquaculture- than for ecotoxicology-related studies (e.g., Forné et al. 2009, 2011; Salas-Leiton et al. 2009; Osuna-Jiménez et al. 2009; Costa et al. 2010b).

The present work aimed at surveying alterations to hepatic cytosolic protein regulation patterns in juvenile Senegalese soles exposed to contaminated sediments collected from an anthropogenic-impacted estuary (The Sado, W Portugal), as a contribution to the understanding of the biological mechanisms underlying exposure to complex mixtures of contaminants, as in natural sediments. It was also intended to screen for protein expression patterns that could be related to sediment contamination. Through a dual arrangement of laboratory and in situ bioassays, it was also aimed at comparing the two assay methodologies.

Methods and materials

Experimental design and procedure

The study area (Fig. 1) consists of a large estuarine basin of high socio-economical importance, impacted by many different sources of pollution, both point and diffuse. The area comprises the city of Setúbal (with an important commercial harbour) plus adjacent suburban areas, and a large heavy-industry belt that includes chemical plants, a thermoelectric power plant, shipyards, ore deployment facilities, a paper mill and others. The estuary is also very important for tourism, local fisheries and maritime transport. To all these pressures it can be added the runoffs from upstream agriculture grounds that may carry pesticides and fertilizers, besides being the river itself an important source of metals since it transverses a pyrite mining region (Cortesão and Vale 1995). In addition, a large part of the estuary is classified as a Natural Reserve. Recent surveys found sediment from the estuary to be moderately contaminated by mixed classes of organic and inorganic substances, however, the observed levels of contamination have been found to induce harmful effects on organisms (see for instance Caeiro et al. 2009; Costa et al. 2009, 2010a and references therein).

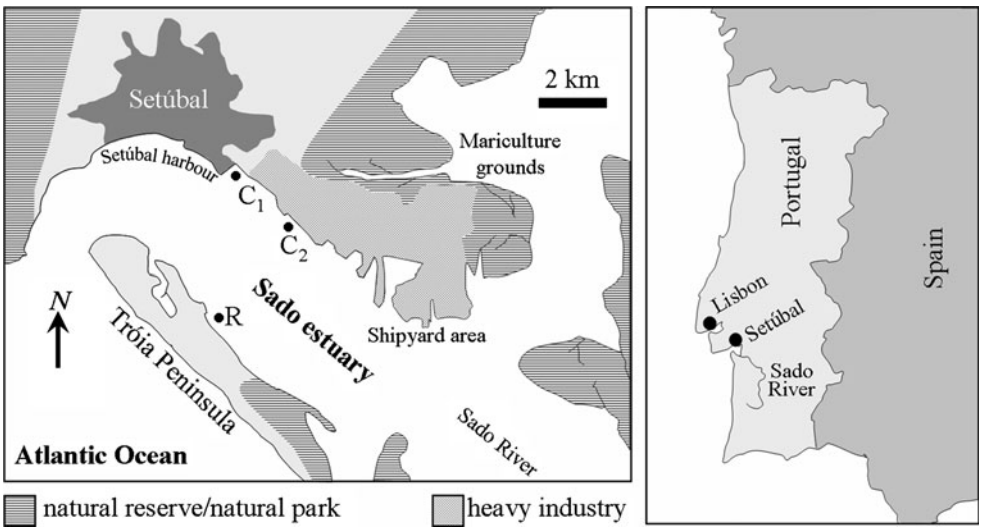

Fig. 1 Location map of the Sado estuary around the city of Setúbal, showing the sediment collection and in situ assay sites (•): R (reference) plus C_1 and C_2 (contaminated), respectively N 38 28.340 W 8 52.056; N 38 30.586 W 8 51.525 and N 38 29.844 W 8 50.444

Juvenile Senegalese soles (standard length = 61.0 ± 8.4 mm; total wet weight = 3.1 ± 1.6 g) were exposed to sediments from three surveyed sites, R (reference), C_1 and C_2 (Fig. 1), through simultaneous laboratory and in situ (field) 28-day bioassays. The three sites were chosen according to information obtained from a previous ERA strategy for the estuary (refer to Caeiro et al. 2009). Fish were hatchery-brood and laboratory-reared and all from the same cohort. The sediments were collected with a grab from each site on May 2007 for physico-chemical characterization and preparation of the laboratory assays. For simplification, exposure to the three sediments will be referred to as tests R, C_1 and C_2.

The laboratory assays were prepared by placing 2 L of fresh sediments in 15 L-capacity white polyvinyl tanks with blunt edges to which 12 L of clean, filtered, seawater was added. The sediments were allowed to settle for 48 h before the beginning of the assays. A recirculation arrangement was adapted to the test tanks, as well as permanent aeration (dissolved $O_2 \approx 55$–60%). Water and air flows were adjusted to avoid sediment disturbance. A weekly 25% water change was done in order to maintain water quality similar to rearing conditions (salinity = 32.1 ± 0.3; pH 8.0 ± 0.1; total ammonia = 1.6 ± 0.6 mg L^{-1}; unionized, toxic, ammonia = 0.04 ± 0.02 mg L^{-1}), with minimal removal of waterborne contaminants and suspended matter. Animals were fed daily with commercial pellets for aquaculture fish (from Sorgal, Ovar, Portugal). Temperature and photoperiod were set at 18 ± 1°C and 12:12 h light:dark, respectively. All tests were performed in duplicate (meaning two tanks per test); with twenty randomly selected soles being allocated per tank. The in situ assays were performed at the location of sediment collection with submerged cages (consisting of a plastic 90 × 90 × 30 cm frame lined by a 5 mm plastic mesh) placed by scuba diving over the bottom, ensuring direct contact of animals to the sediment. Each cage was divided in two equal-sized compartments regarded as replicates. Each compartment held twenty randomly selected fish.

Sampling occurred after 14 days of exposure. The animals exposed in situ were transported alive to the laboratory where, as laboratory-tested fish, were euthanized by cervical sectioning before dissection and liver excision. Animals sampled before the beginning of the bioassays (T_0 fish) were taken as the calibrator group for subsequent analyses.

Sediment characterization

Sediments were analysed for total inorganic (element) and organic contaminants from dried samples. The non-metal selenium (Se), the metalloid arsenic (As) and the metals cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn), were quantified by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Elemental X-Series equipment, after mineralization with acids (HCl, HNO_3 and HF) in closed Teflon vials according to Caetano et al. (2007). Total mercury (Hg) was determined by atomic absorption spectrometry (AAS) according to Costley et al. (2000), after sample pyrolysis (750°C in an oxygen atmosphere) in a combustion tube attached to an AMA-254 mercury analyzer (Leco). The same protocols were applied to the certified reference sediments MESS-2and PACS-2 (National Research Council, Canada) plus MAG-1 (US Geological Survey, USA) to verify the adequateness of the procedure and the measurements were found within the certified range.

Sediment PAHs (comprising a total of seventeen 3- to 6-ring compounds) were quantified by gas chromatography-

mass spectrometry (GC–MS) after Soxhlet extraction with an acetone + hexane (1:1 v/v) mixture (Martins et al. 2008). Organochlorines, namely eighteen polychlorinated biphenyl (PCB) congeners and the dichloro diphenyl thichloroethane (DDT) pesticide (with total DDT meaning the sum of concentrations of *pp*'DDT plus its main metabolites *pp*'DDE and *pp*'DDD) were determined by GC with electron capture detection (GC-ECD) after Soxhlet extraction with *n*-hexane and column fractioning (Ferreira et al. 2003). The procedure was validated by analysis of the SRM 1941b reference sediment (National Institute of Standards and Technology, USA) and the obtained values were found within the certified range.

The sediments' redox potential (Eh) was measured immediately after collection with an electrode apparatus equipped with a platinum electrode with an Ag/AgCl reference electrode. Sediment total organic matter (TOM) was extrapolated from total organic carbon loss-on-ignition after combustion at 500°C for 5 h. Fine particle fraction (FF), particle size <63 μm, was determined after disaggregation with pyrophosphate and hydraulic sieving.

Proteomic analysis

The alteration to hepatic cytosolic proteins' expression pattern was determined by two-dimensional electrophoresis (2-DE) as developed by Romero-Ruiz et al. (2006) and Montes-Nieto et al. (2007) and described by Salas-Leiton et al. (2009) and Costa et al. (2010b) for the species, with a few modifications. For each experimental condition (exposure either in laboratory or in the field to the three sediments, plus T_0 animals), frozen (−80°C) liver samples from four randomly selected individuals per experimental condition (two per replicate) were pooled and homogenized in liquid nitrogen. Crude protein extraction was achieved by placing ≈100 mg of each liver homogenate in 20 mM Tris–HCl buffer (pH 7.6) with 0.5 M sucrose and 0.15 M KCl and complemented with μL ml^{-1} 2-hydroxyethyl disulfide (HED) as a reducing agent. HED was employed after preliminary trials showed it to very significantly reduce streaking in second-dimension gels when compared to the more commonly used dithiothreitol (DTT). One mM phenylmethylsulfonyl fluoride (PMSF), 6 mM leupeptin and 100 μL mL^{-1} of Protease Inhibitor Cocktail (Sigma) were added to the extraction buffer as protease inhibitors. After grinding with a pestle, samples were centrifuged for 1 min to remove the lipid supernatant, followed by another 10 min centrifugation (14,000×*g* at 4°C). Samples were then treated with 500 U mL^{-1} benzonase endonuclease for 30 min (at room temperature), followed by centrifuging at 102,000×*g* for 1 h (at 4°C) to precipitate the remaining non-peptide material. Total protein was determined according to Bradford (1976) so each immobilized pH gradient (IPG) strip (18 cm, pH 4–7; from GE Healthcare) could be loaded with 100 μg of protein. The pH gradient was selected according to previous proteomic surveys on *S. senegalensis* liver (Costa et al. 2010b). Three IPG strips were prepared per each experimental condition, meaning all subsequent steps were performed in triplicate. Strips were incubated (30 min at room temperature) in pH 4–7 IPG buffer (GE healthcare) complemented with 7 M urea, 2% w/v of the non-ionic detergent CHAPS, 60 μL ml^{-1} of the HED reducing agent, plus ≈1% w/v bromphenol blue. Strips were then allowed to passively rehydrate (6 h, 20°C) and afterwards subjected to isoelectric focusing (IEF) on a Protean IEF apparatus (Bio-Rad) for first-dimension protein separation (according to isoelectric point). The second dimension separation (by molecular weight) was achieved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 18 × 18 cm 12.5% acrylamide/*bis*-acrylamide gels. Prior, IPG strips were equilibrated in 1.5 M Tris–HCl buffer (pH 8.8) to which were added 6 M urea and 2% SDS as denaturing agents; 60 μL ml^{-1} HED; 3% glycerol and approximately 1% w/v bromophenol blue. Equilibration of IPG strips was followed by a treatment with 25 mM iodacetamide (IAA) to block sulphydryl groups. Electrophoresis was then run at constant wattage (10 W per gel) in a DodecaCell Plus device (Bio-Rad). The SigmaMarker wide-range protein ladder (Sigma–Aldrich) was used as molecular weight (MW) standard. Gels were afterwards stained with the Sypro Ruby fluorescent dye (Bio-Rad) and imaged with a Bio-Rad FXImager laser scanner. All image analyses and determination of protein regulation factors were done with ProteomWeaver (Bio-Rad).

The protein spots in gels were selected for identification according to the criteria of a significant statistical difference in spot intensity (given by the Mann–Whitney *U* test) between at least one experimental test to T_0 gels (used as the calibrator group for statistical purposes) plus a minimum of 50% up- or downregulation relative to T_0 animals (calibrator group), to ensure contrast between treatments (e.g., Montes-Nieto et al. 2007; Costa et al. 2010b). The spots that met the criteria were excised from gels and treated with DTT and IAA prior to digestion with porcine trypsin. The digestion products were then preliminarily sequenced by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in a Voyager De-PRO apparatus (Applied Biosystems) to determined the quality of the samples and eliminate background from trypsin self-proteolysis. The samples were afterwards subjected to de novo sequencing by capillary liquid chromatography electrospray ionization ion trap tandem mass spectrometry (capLC-ESI-ITMS/MS) using a LTQ system (ThermoFisher Scientific). Peptide search was performed using the Protein–Protein Blast

2.2.20 software (Altschul et al. 1997) to contrast results to chordate, actinopterygian and pleuronectiform taxa peptide sequences existing in the NCBI (National Centre for Biotechnology Information of the USA) All-Non Redundant Protein Sequence database. Protein identification was validated according to the criteria of highest score, lowest *e*-value, number of matched peptides and representation in the prospected database sets.

Statistical analysis

The statistical significance of the differences between spot intensities were surveyed by the non-parametric Mann–Whitney *U* test considering the small n ($n = 3$) for parametric analyses. Statistics were performed using the software Statistica (Statsoft). A significance level of $\alpha = 0.05$ was set for all analyses. The relative abundance of the identified proteins was assessed as the percentage of spot intensity relatively to T_0 fish. Cluster analysis on protein expression data was performed with the software DAnTE (Polpitiya et al. 2008).

Results

Sediment characterization

As shown in Fig. 2, the reference sediment (R) was confirmed to be the least contaminated, as well as the least anoxic (Eh = −140 mV) and the most sandy (FF = 2%, TOM = 23%). The two most contaminated sediments revealed distinct patterns of contamination; although both contained a blend of organic and inorganic contaminants. Sediment C_1 (the closest to the city of Setúbal, Eh = −300 mV, FF = 96%, TOM = 10%) was the most contaminated by metals while sediment C_2 (located off the city's heavy industry area, Eh ≈ −300 mV, FF = 76%, TOM = 7%) was mostly contaminated by PAHs and organochlorines even though the levels of sediment organic contaminants were similar between C_1 and C_2. In general, these results are in accordance with previous characterization of the sites (Costa et al. 2009) but site C_2 revealed an increase in metal and organochlorine concentrations. By comparing with previous research in the area, the

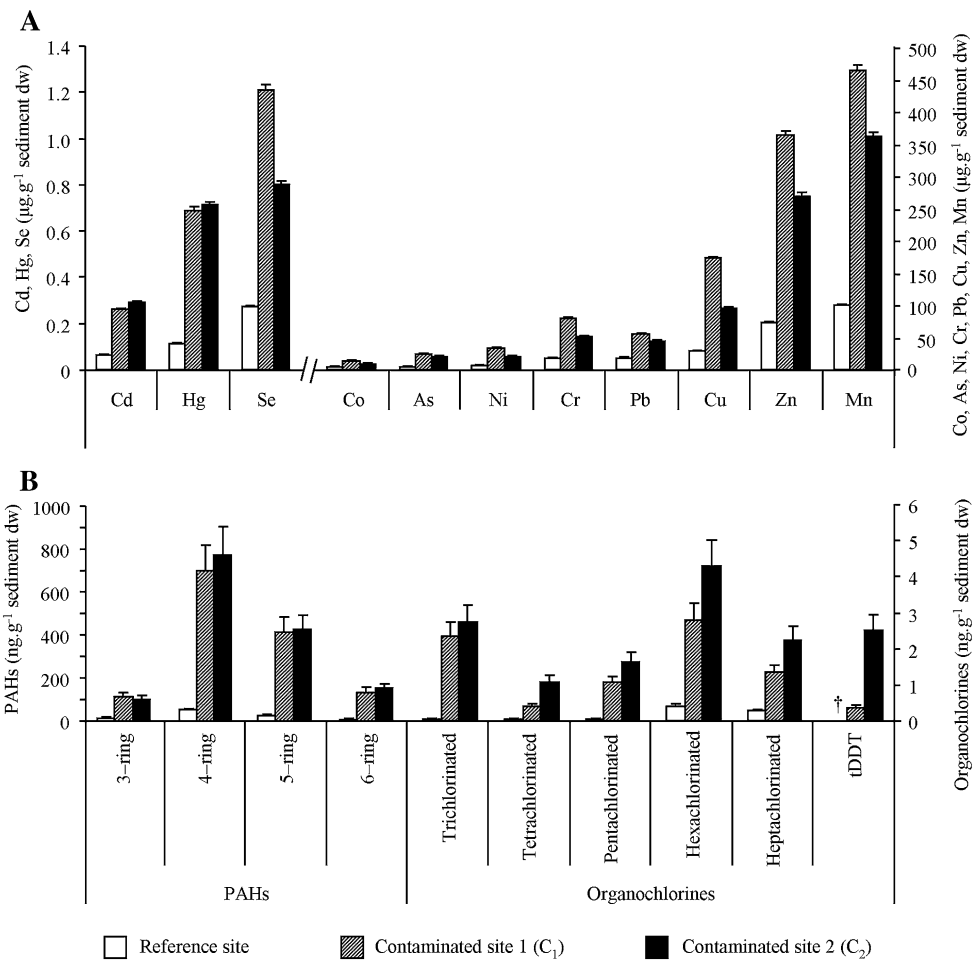

Fig. 2 Concentrations of element (**a**) and organic contaminants (**b**) in the surveyed sediments R (reference), C_1 (most contaminated by metals) and C_2 (most contaminated by organic substances. † Means below detection limit. The level of Hg in the sediments refers to total Hg (organic plus inorganic species)

sediments can be globally regarded as moderately contaminated (Costa et al. 2010a).

Besides the levels of As, Cd and total Hg, similar between the two contaminated sediments, the surveyed elements' concentrations were consistently higher in sediment C_1 (Fig. 2a). The most noticeable differences between C_1 and C_2 were obtained for Cu ($\approx$1.8 fold), Cr and Ni ($\approx$1.6 fold for both). Compared to the reference sediment (R), Cu and Hg levels were higher in contaminated sediments by approximately sixfold. Four- and five-ring compounds represented the largest majority of PAHs in all sediments, representing $\approx$75–80% of total PAH concentrations (Fig. 2b). Fluoranthene and pyrene had the highest concentrations, with 315.7 and 263.2 ng g^{-1} sediment dw in sediment C_1 and 345.2 and 286.3 ng g^{-1} sediment dw for C_2, respectively. For all sites, the phenanthrene/anthracene and fluoranthene/pyrene ratios were >1 and <10, respectively, indicating mostly pyrolytic origin (combustion-derived) of PAHs rather than petrogenic [i.e., derived from fossil fuels (Budzinski et al. 1997)]. Hexachlorinated PCBs were the most representative PCBs in all sediments, ranging between $\approx$35 and 50% of total PCB concentrations (Fig. 2b). The highest concentration for this specific group was found for PCB 153, in sediment C_2 (1.23 ng g^{-1} from a total of 4.29 ng g^{-1} sediment dw for hexachlorinated PCBs). The overall most concentrated PCB was PCB 26, in both C_1 and C_2 sediments, with 1.8 and 2.0 ng g^{-1} sediment dw, respectively (not detected in the reference sediment).

Proteomic analyses

The 2-DE analyses yielded forty-one cytosolic protein spots (out of $\approx$3,000 detectable spots in gels) that met the criteria of consistent 50% up- or down-regulation relatively to the calibrator group. The number of up-regulated/down-regulated protein spots for the laboratory tests was 15/4, 7/1 and 7/1 for tests R, C_1 and C_2, respectively and 17/6, 15/7 and 19/6 for the in situ assays. From these forty-one spots, 19 could be matched to known proteins by contrasting the ESI-ITMS/MS results to nrNCBI database after quality checking by MALDI-TOF (Fig. 3). All peptides potentially resulting from keratin contamination were excluded from subsequent analyses. Due to the reduced sequencing of the species' genome and peptidome, only two proteins could be directly matched to *S. senegalensis*, namely β actin and the trypsin precursor trypsinogen 1c. Still, peptide matching yielded a low *e* value, ranging between the scales of 10^{-6} to 10^{-20}. The best matched protein was the proteasome subunit β type 9 with a score of 94.9 and an *e* value of 6×10^{-20} (Table 1). The identified proteins take part in multiple cellular processes, from cytoskeleton (β-actin), oxidative stress response (like 1-cys peroxiredoxin) to enzymes involved in glycolysis (such as enolases and lactate dehydrogenase) and specialised aminoacid metabolism (glutamine synthetase and glycine-*N*-methyltransferase) to proteolysis (cathepsin, trypsin/trypsinogen, proteasome subunits) and xenobiotic catabolism, as for some oxidoreductase enzymes (Table 2).

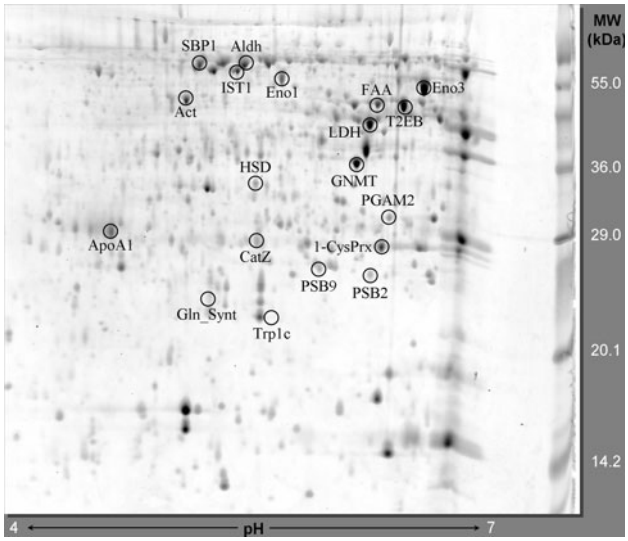

Fig. 3 Representative second dimension SDS–PAGE gel image showing the differentially expressed spots that were identified by ITMS/MS

Only glutamine synthetase and trypsinogen 1c were found significantly downregulated (below 50%) relatively to T_0 fish, in laboratory exposure to sediment C_1 and all field tests, respectively. All other cases represent peptides where regulation was either found to be unchanged or increased (above the 150% threshold compared to T_0 animals). The highest upregulation was obtained for trypsinogen (378%) in C_1-tested fish in the laboratory, followed by the transcription initiation factor IIE subunit β in laboratory assayed fish exposed to the reference sediment, R (298%). Laboratory tests with sediments C_1 and C_2 result in a reduced number of proteins that had their regulation patterns changed compared to all field-assayed animals and animals exposed to sediment R in either type of assay.

Comparing the regulation patterns of C_1- and C_2-tested fish in either type of assay to the respective reference test (exposure to the clean sediment R in the laboratory or in situ), it was observed that only peroxiredoxin and trypsinogen were significantly upregulated in laboratory tested fish (C_1 and C_2 tests), as well as lactate dehydrogenase (in test C_1 only). Regarding the in situ experiment, upregulation was observed for aldehyde dehydrogenase, enolase 3, fumarylacetoacetase, glutamine synthetase and lactate dehydrogenase, mostly linked to energy production (via the glycolytic pathway) and aminoacid metabolism processes. Exposure to sediment C_1 (most metal-contaminated) in the

Table 1 Cytosolic protein identification summary after de novo sequencing using ESI-ITMS/MS and peptide sequence database search with Protein–Protein Blast and correspondent regulation factors (percentage of relative regulation ± standard deviation) after the 14-day exposure to the reference (R) and contaminated sediments (C_1 and C_2)

Protein	Abbreviation	Uniprot accession	Database taxa[a]	Score	*e* value	Matched peptides	Protein regulation factor					
							Laboratory assay			In situ assay		
							R	C_1	C_2	R	C_1	C_2
17-beta hydroxysteroid dehydrogenase	HSD	Q7T2J0	1,2,3	83.3	4×10^{-17}	4	**203.2 ± 14.2**	138.9 ± 18.1	144.9 ± 6.6	**239.3 ± 8.9**	**259.5 ± 14.2**	**178.1 ± 11.2**
1-*Cys* peroxiredoxin	1-CysPrx	Q8AWH6	1,2,3	41.8	1×10^{-6}	2	71.7 ± 7.1	127.7 ± 27.6	**150.6 ± 19.3**	89.1 ± 18.5	120.1 ± 11.9	77.8 ± 17.2
Aldehyde dehydrogenase	Aldh	Q98TM9	1,2,3	63	6×10^{-13}	5	**203.4 ± 4.9**	106.9 ± 10.3	101.6 ± 5.9	**163.7 ± 20.2**	**163.0 ± 20.3**	**211.0 ± 8.8**
Apolipoprotein A-1	ApoA1	O42363	1,2,3	60	4×10^{-10}	3	139.2 ± 9.1	136.2 ± 15.2	113.9 ± 15.5	**158.0 ± 18.7**	**154.6 ± 7.5**	90.0 ± 11.3
β Actin	Act	Q1HHC7	1,2,3	71	2×10^{-15}	8	139.1 ± 12.8	95.8 ± 6.5	119.1 ± 13.8	**225.3 ± 15.3**	140.6 ± 16.6	**159.3 ± 6.6**
Biphosphoglycerate mutase 2	PGAM2	B5XD74	2,3	52	1×10^{-7}	3	**161.8 ± 11.4**	108.6 ± 3.0	129.4 ± 25.2	103.7 ± 9.2	105.8 ± 15.2	87.9 ± 19.4
Cathepsin Z	CatZ	Q58HF4	1,2,3	77	8×10^{-15}	1	117.1 ± 4.9	99.5 ± 23.2	101.8 ± 55.1	**167.3 ± 27.5**	141.9 ± 17.5	**196.0 ± 10.4**
Enolase 1	Eno1	C0H878	2,3	57.5	3×10^{-9}	4	139.2 ± 9.1	136.2 ± 15.2	113.9 ± 15.5	**158.0 ± 18.7**	**154.6 ± 7.5**	90.0 ± 11.3
Enolase 3	Eno3	Q568G3	2,3	68.1	2×10^{-12}	1	**165.7 ± 6.1**	80.7 ± 16.0	72.7 ± 29.7	127.8 ± 8.2	150.7 ± 6.5	147.7 ± 20.2
Fumarylacetoacetase	FAA	Q803S0	2,3	46	7×10^{-6}	1	**193.9 ± 2.3**	120.9 ± 2.8	108.7 ± 6.3	97.8 ± 13.9	122.2 ± 14.0	**193.4 ± 4.9**
Glutamine synthetase	Gln_Synt	Q4RVF3	1,2,3	72.4	2×10^{-13}	2	85.9 ± 19.5	**47.6 ± 23.2**	63.9 ± 21.8	120.5 ± 1.6	97.2 ± 6.7	84.4 ± 8.5
Glycine *N*-methyltransferase	GNMT	Q6P607	2,3	71	2×10^{-13}	4	140.2 ± 5.8	129.7 ± 8.1	130.3 ± 6.2	122.4 ± 3.2	137.3 ± 6.6	**161.7 ± 9.1**
Malate/*L*-lactate dehydrogenase	LDH	Q7T3D9	2,3	75.3	1×10^{-14}	2	129.7 ± 8.7	**168.2 ± 17.8**	128.6 ± 15.3	**158.0 ± 16.0**	**181.7 ± 7.1**	**152.3 ± 16.0**
MAPK activating protein PM28	IST1	P53990	2,3	76.3	2×10^{-14}	3	149.0 ± 9.8	**154.6 ± 27.1**	125.8 ± 16.5	**203.3 ± 10.6**	139.2 ± 10.2	**214.4 ± 13.4**
Proteasome subunit β type 2	PSB2	Q6DHI9	2,3	52.4	9×10^{-8}	2	**244.8 ± 5.5**	144.8 ± 11.9	121.9 ± 16.3	**231.9 ± 14.8**	**221.9 ± 17.5**	**236.2 ± 11.8**
Proteasome subunit β type 9	PSB9	Q9DD33	1,2,3	94.9	6×10^{-20}	2	126.5 ± 7.9	79.9 ± 23.4	94.8 ± 32.6	**155.3 ± 7.2**	**162.4 ± 13.9**	114.7 ± 9.0
Selenium binding protein 1	SBP1	Q6PHD9	2,3	69.7	1×10^{-12}	3	**272.7 ± 10.9**	**195.3 ± 18.9**	**216.0 ± 13.7**	**234.5 ± 6.1**	**230.2 ± 1.8**	127.6 ± 26.0
Transcription initiation factor IIE subunit β	T2 EB	T2 EB	2,3	56.6	5×10^{-9}	2	**298.2 ± 9.1**	126.0 ± 6.2	137.6 ± 20.6	**228.8 ± 5.1**	**265.4 ± 18.2**	**205.1 ± 8.3**
Trypsin/Trypsinogen 1c	Trp1c	A7VMR6	1,2,3	54.5	2×10^{-10}	2	91.8 ± 9.0	**378.0 ± 8.1**	**165.8 ± 4.9**	**48.0 ± 5.9**	**23.8 ± 36.9**	–[b]

Regulation factors were estimated taking T_0 fish (calibrator group) as baseline reference. Boldface numbers indicate significant changes to regulation patterns (over T_0 fish) according to the imposed 50% up- or -downregulation threshold (Mann–Whitney *U*, $p < 0.05$)

[a] nrNCBI database taxa from which peptide matched was obtained: 1-Order Pleuronectiformes; 2-Class Actinopterygii; 3-Phylum Chordata

[b] No expression was observed in any of the triplicate gels of the experimental condition

Table 2 Main function of the identified proteins

Function class	Protein	Activity
Aminoacid metabolism	Fumarylacetoacetase	Metabolism of aromatic aminoacids
	Glutamine synthetase	Glutamine biosynthesis (catalyses the ammonia-glutamate bond)
	Glycine *N*-methyltransferase	Methionine metabolism
Cell cycle progression	MAPK activating protein PM28	Involved in cell division, probably in cytokynesis
Cell structure	β actin	Key component of the cytoskeleton
Energy production	Biphosphoglycerate mutase 2	Glycolysis
	Enolase 1 and 3	Glycolysis and gluconeogenesis
	Malate/*L*-lactate dehydrogenase	Involved in anaerobic energy production (fermentation)
Gene transcription	Transcription initiation factor IIE subunit β	Part of the RNA polymerase initiation complex
Intracellular signalling	Selenium binding protein 1	Unknown. May be involved in xenobiotic sensing in cytoplasm and in anti-carcinogenic mechanisms
Lipid transport	Apolipoprotein A-1	Promotes cholesterol efflux from cells
Oxidoreductase enzymes	1-*Cys* peroxiredoxin	Scavenges oxidative radicals. Evidence for function in phospholipid turnover
	17-beta hydroxysteroid dehydrogenase	Androgen and oestrogen metabolism and biosynthesis.
	Aldehyde dehydrogenase	NAD-dependent oxidant catalyst
Proteolysis	Trypsin/trypsinogen (trypsin precursor) 1c	Serine protease (protease S1 family)
	Cathepsin Z	Cysteine protease (protease C1 family)
	Proteasome subunits (β) types 2 and 9	Component of the proteasome (macropain) multicatalytic proteinase complex (ATP-dependent)

laboratory resulted in the greatest percentage of downregulated proteins compared to the reference test, with a total of twelve out of nineteen proteins being underexpressed, followed by the C_2 laboratory test, with seven (Fig. 4).

Discussion

When compared to initial state soles (T_0 fish), all tests, including exposure to the reference sediment (R), elicited some degree of hepatic proteome changes (Table 1). However, the results from exposure to sediment R should represent a baseline of metabolic disturbance caused by the bioassays per se (e.g., from having changed the animals' environment). When the proteomic responses of animals exposed to contaminated sediments are contrasted to R-tested fish, an altered pattern of regulation is revealed that can be explained by differences in the sediments' contamination levels. The differences between laboratory and field assayed animals, on the other hand, may be partially explained by the differential bioavailability of sediment contaminants and unknown environmental parameters affecting in situ tests.

In previous laboratory bioassays the hypothesis of increased bioavailability of contaminants as consequence of sediment disturbance caused by collection, handling and animal activity has already been discussed (Costa et al. 2009, 2010a). Aquatic sediments are complex matrices that sorb both hydrophilic and apolar substances. Disturbance can greatly alter the sediments steady-state, e.g., through anoxic–oxic shifts, favouring contaminant desorption (Eggleton and Thomas 2004). However, metallic and organic contaminants may be differentially released from sediments, since the latest are the most insoluble and reveal complex desorption from sediments, depending on the toxicant's class and the characteristics of the sediment's organic matter (Kukkonen et al. 2003). Metals, on the other hand, tend to form complexes with other mineral substances and their release to water may be greatly affected by pH and redox status changes (Geffard et al. 2005; Du Laing et al. 2009). In fact, redox potential (Eh) and sediment pH are interlinked, since oxidation, for example driven by sediment disturbance and subsequent re-oxygenation of anoxic layers, leads to acidification, which on its turn favours release of metals from sediments and avoids its deposition (refer to Du Laing et al. 2009, for a review). It is likely that inorganic and organic substances were differentially released from the sediments, a phenomenon that should be of greater relevance in the field, where disturbance was probably less of an issue. Also, caged fish were scavenging sediments for preys that may have accumulated contaminants themselves, whereas in the laboratory assays

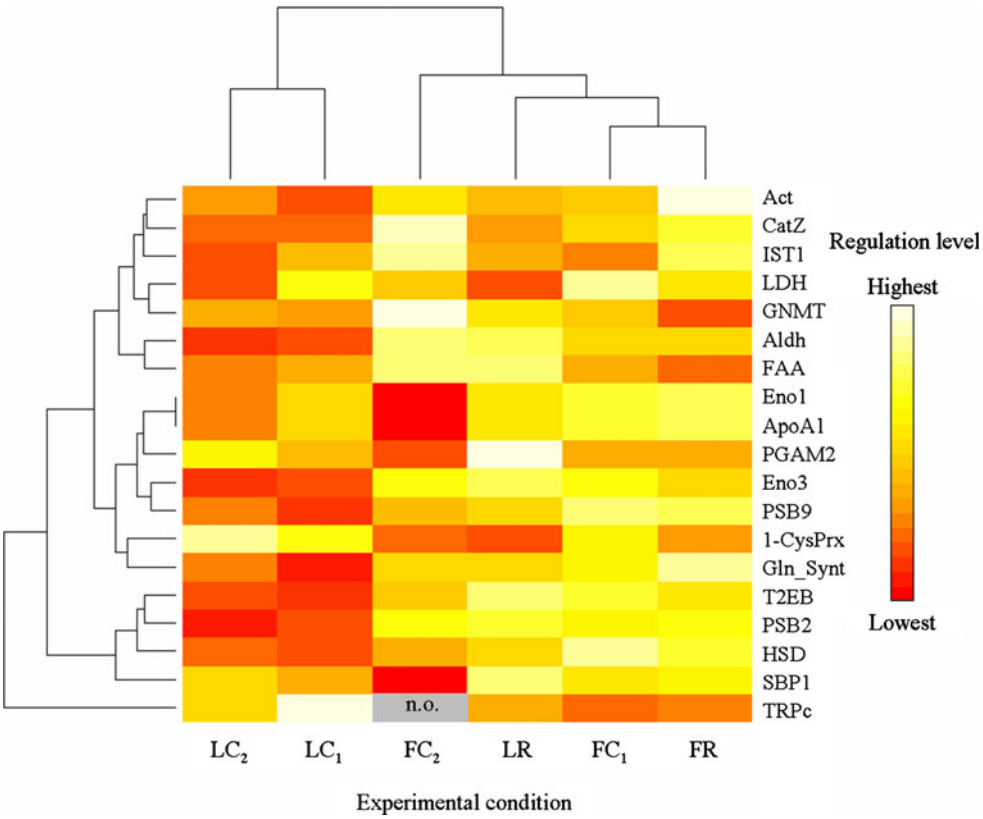

Fig. 4 Cluster analysis on protein regulation factors and experimental tests. Complete linkage was employed as amalgamation rule and Euclidean distances as metrics. [n.o.] means no expression observed. L and F indicate laboratory and in situ (field) exposure, respectively, to sediments R (reference), C_1 and C_2 (contaminated)

(where the fish were fed with commercial pellets) the main exposure route was likely through gills.

Compared to the reference test, laboratory exposure to both contaminated sediments caused up-regulation of 1-CysPrx, a peroxiredoxin, and TRPc, a trypsin peptidase precursor (Fig. 5). Peroxiredoxins are proteins mainly involved in the scavenging of oxidative radicals in the presence of thioredoxins, with evidence for other functions

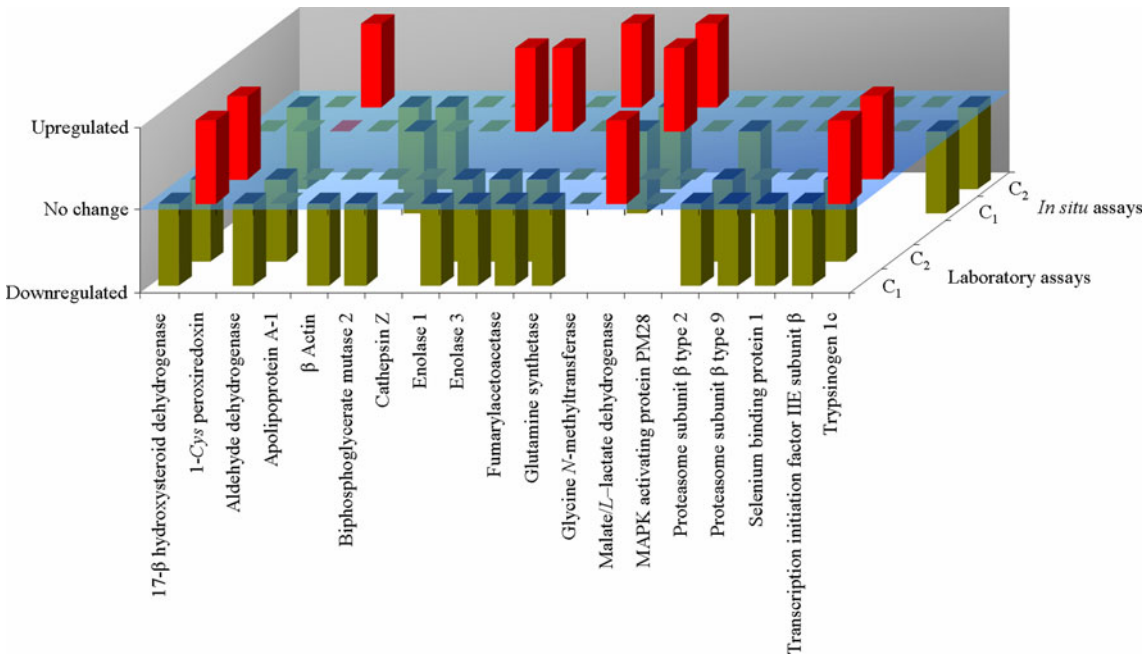

Fig. 5 Altered protein regulation patterns in C_1- and C_2-tested fish, relative to respective reference test (exposure to sediment R). Columns indicate significantly up- or-down regulated proteins (Mann–Whitney U, $p < 0.05$)

such as phospholipid catabolism, by containing two distinct active sites, one similar to the glutathione peroxidase active site and another similar to a Ca^{2+}-independent phospholipase (Chen et al. 2000), which might contribute to its known role in the protection of membrane integrity (Manevich et al. 2002). Peroxiredoxins are regarded as important anti-oxidant enzymes that can be induced by various stressors. There is evidence, at transcript and protein levels, that Cd induces peroxiredoxins in flatfish liver (Sheader et al. 2006; Costa et al. 2010b). Although David et al. (2007) also related a peroxiredoxin gene overexpression to increased estuarine pollution in oysters (in this case, the proteins' isoform 6), there is surprisingly little information relating this family of proteins to environmental contamination. Trypsinogen (a zymogen), in its turn, is the inactive translation product of trypsin, a serine protease that, besides its role in digestion, has been found linked, either by suppression or induction, to a number of disorders. Although the exact mechanisms and importance of trypsinogen regulation against toxicological challenge remain unclear, some authors reported that trypsinogen gene expression has some potential as a biomarker in fish due to its conspicuous underexpression upon exposure to an organophosphorous pesticide (Sinha et al. 2010). Also, some evidence exists that exposure to Cd and to the combination of Cd plus the PAH benzo[a]pyrene (B[a]P), but not B[a]P alone, may induce trypsinogen and 1-CysPrx in *S. senegalensis* (Costa et al. 2010b). Altogether, this information may indicate that in the laboratory studies both metallic and organic toxicants were released (as a result of increased bioavailability), whereas organic contaminants exerted the most compromising effects in situ. The regulation pattern of glycine *N*-methyl transferase (GNMT), whose overexpression has been found to reduce B[a]P cytotoxicity in human cell-based bioassays (Lee et al. 2006), also supports the hypothesis of the differential bioavailability of organic and metallic contaminants. Other authors found GNMT transcription to be elevated as a consequence of exposure to B[a]P in *Fundulus heteroclitus* embryos, discussing the role of GNMT as a defence strategy due to its role in DNA methylation (Fang et al. 2010). Accordingly, this enzyme was most upregulated in field exposure to sediment C_2 (the most contaminated by organic substances, especially PAHs), and significantly upregulated for both C_1 and C_2 tests relative to the reference, which was not observed in the laboratory assays.

Little information exists regarding direct action of toxicants in the inhibition of gene transcription per se. The downregulation of the transcription initiation factor T2 EB (also termed transcription factor IIE, TIF IIE subunit β) in fish exposed to the contaminated sediments in the laboratory (Fig. 5) may aid explaining the overall protein downregulation in fish subjected to this test. Other authors already reported that genes and proteins related to transcription control can be affected by exposure to oxidative substances; as for instance the ATF7, a leucine zipper (bZIP) transcription factor (Weigel et al. 2002; Montes-Nieto et al. 2010). In spite of the importance of TIF IIE to the RNA polymerase II initiation complex (Peterson et al. 1991), no information was found regarding the interference of toxicants on its regulation. It is plausible though, that the impairment of gene translation significantly affected general metabolic processes and some of the response and defence mechanisms towards exposure to toxicants in fish exposed to sediments C_1 and C_2.

The function and mechanism of action of selenium-binding proteins (SBPs) are not yet fully understood. These $\approx$54 kDa proteins were found to be induced by organic contaminants such as PAHs (Ishida et al. 1998) and organochlorines (Ishii et al. 1996). It is therefore not clear why this protein appeared downregulated after exposure to sediment C_1, in the laboratory, comparatively to reference (R-tested) animals. Interestingly, downregulation of SBP1 has been found linked to carcinogenesis in mammal models and it is suspected that this protein mediated ROS (reactive oxygen species) -induced apoptosis of neoplasic or pre-neoplasic cells (for instance Pohl et al. 2009). In accordance, some evidence exists on SBP1 downregulation by oxidative stress (Giometti et al. 2000). Altogether, it is likely that SBP1 has some role in defence mechanisms against injury. Its downregulation may thus have compromised responses in fish exposed to contaminated sediments.

Tissue clean-up of damaged cells (e.g., through apoptosis) and cell proliferation can be an important part of organ recovery following toxicant-induced stress. Some proteins taking part in these functions have been found to be closely linked (Fig. 4), namely the structural protein β actin, IST1 (linked to cell cycle progression) and a cathepsin cysteine protease, CatZ, the latest belonging to a class that is known to intervene in programmed cell death (Chwieralski et al. 2006). The more pronounced downregulation of such proteins in laboratory exposure to sediments C_1 and C_2 is yet another line of evidence of stress-response impairment that can be related to increased contaminant bioavailability during the laboratory assays. It should be noted, though, that CatZ and IST1 were the only proteins whose regulation in fish exposed to contaminated sediments did not exhibit any significant variation above or below the 50% threshold compared to the reference test, in either type of bioassays (Fig. 5). It is possible that deregulation of these enzymes is partly linked to experimental stress.

The proteasome is an ATP-dependant proteolytic complex important in the elimination of cytoplasmic proteins marked for destruction (by ubiquitination) and therefore has a key regulatory process after chemical-induced injury. It should be noted, though, that other peptidases, like

trypsin, also have an important role in cellular and tissue clean-up after damage although the functions and energy demands are dissimilar. Serine proteases such as trypsin are also suspected to have an important role in apoptosis (Stenson-Cox et al. 2003). Nevertheless, trypsinogen regulation was affected in a different manner by exposure to the contaminated sediments than the proteasome subunits, with no relation being found between these two very distinct proteases. Impairment of the proteasome activity has been linked to a series of disorders, from neurodegeneration (Bedford et al. 2008) to some forms of hepatocellular degeneration, such as the keratin aggresome-based Mallory-Denk bodies (Harada et al. 2008). Even though research on proteasome induction and activity is currently under a spotlight for its potential application in anti-cancer treatments, specific information regarding the effects of toxicants is scarce. Nevertheless, Biales et al. (2011) discovered proteasome subunit downregulation in a proteomic survey on fish exposed to pesticides and Bardag-Gorce et al. (2006) found activity and regulation of proteasome to be downregulated in human cultured liver cells exposed to ethanol as a consequence of ROS production, which is consistent with the present findings even though it is not clear why PSB 2 and 9 relative regulation appeared to be little correlated (Fig. 4). Still, proteasome subunit down-regulation in fish exposed to the contaminated sediments (especially C_1, the most contaminated by metals), when compared to the reference condition likely resulted in unbalanced protein regulation and recycling.

Energy production enzymes were found deregulated by exposure to contaminated sediments, namely PGAM2, Eno1 and Eno3, which take part in the glycolytic pathway, and LDH, involved in anaerobic respiration. Increased regulation of glycolytic pathway enzymes has been found to occur in animals exposed to toxicants, e.g., in the liver of mice dwelling in metal-polluted sites (Montes-Nieto et al. 2007) and in the kidney of rats exposed to bromates (Ahlborn et al. 2009). However, Biales et al. (2011) reported increased regulation of glycolytic enzymes in the brain of fathead minnows (*Pimephales promelas*) as a consequence of exposure to individual pesticides and a down-regulation as a result of co-exposure. Other authors found that Cd may down-regulate glycolytic enzymes in fish liver (*Micropterus salmoides*) with evidence for an inverse effect resulting from exposure to the PAH phenanthrene (Sanchez et al. 2009). It is likely that exposure to the contaminated sediments altered the respiratory metabolism, although with different outcomes resulting from the laboratory and field bioassays, either due to differential bioavailability or unknown factors. As before, it can be suspected that laboratory-exposed fish were more affected by co-exposure to metallic and organic contaminants, impairing the regulation of energy production enzymes. The resulting unbalance of the respiratory pathway can aid in explaining why the anti-oxidant 1-CysPrx was up-regulated in fish exposed to the contaminated sediments during the laboratory assays, since disruption of aerobic respiration can cause a net loss of available ATP and an increase in ROS (e.g., Lemasters and Nieminen 1997). Furthermore, impairment of glucose metabolism increases oxidative stress since the cellular pool of the anti-oxidant NADPH (co-factor to glutathione reductase) is chiefly maintained through the pentose phosphate pathway (e.g., Cosentino et al. 2011). It should also be noticed that unbalance of the oxidative status compromises glucose and energy production metabolism since many reactions are redox-dependent. Also, it has been hypothesized that down-regulation of glycolytic enzymes and up-regulation of key gluconeogenic enzymes modulates energy metabolism to produce sufficient reducing power for increased antioxidant needs in response to pollution (Montes-Nieto et al. 2007). In fact, 1-CysPrx was found allocated in the same cluster as respiration-related enzymes (Eno 1 and 3 plus PGAM2), together with glutamine synthetase and the lipid transporter ApoA1, related to baseline cellular processes (Fig. 4). Only the allocation of PSB9 within this group remains unclear. Interestingly, PGAM2 displayed an almost opposite trend in regulation compared to the enzyme LDH, intervening in the pyruvate fermentation to lactate therefore recycling NADH back to NAD (Fig. 5), which may indicate that impairment of aerobic production of ATP increased the demand for anaerobic-produced energy. Deregulation of other basal cell function enzymes, such as aminoacid metabolism and cell progression, has been described in several other works with fish liver or liver-derived cell lines (e.g., Malécot et al. 2009, in a study with a microcystin toxin; Kling and Förlin 2009, on fish exposed to brominated flame retardants; Evrard et al. 2010, on fish exposed to mixed herbicides). Deregulation of basal cellular functions constitutes the "disturbance" phase of the stress model discussed by other authors, during which organisms suffer a general imbalance that may lead to either a phase when appropriate responses are finally triggered or to biological failure if stress occurs too fast and/or is too strong to permit some form of adaptation (Steinberg et al. 2008). It is likely that laboratory-tested soles, exposed to more bioavailable contaminants, suffered more pronouncedly the effects of metabolic imbalance and failed to resolve adequate responses to toxicity.

Three oxidoreductase enzymes (HSD, LDH and Aldh) were deregulated by exposure to the contaminated sediments, especially in laboratory-assayed fish, where, with the exception of LDH, the proteins 1-CysPrx, HSD, Aldh plus Eno 1 and 3 were deregulated in fish exposed to both C_1 and C_2 compared to R-tested animals. Only 1-CysPrx was in fact up-regulated when compared to the reference

treatment. Regarding the in situ assays, Aldh alone (in C_2-tested animals) was significantly up-regulated compared to R-tested fish. Dehydrogenases, including HSD, LDH and Aldh (which are NAD/NADP dependent enzymes), are important phase I detoxification enzymes (Oppermann and Maser 2000). The Aldh enzyme was observed to be very significantly upregulated in field-exposed fish to sediment C_2 (most contaminated by organic compounds), which again may indicate that organic contaminants caused a more pronounced effect in situ. Aldehyde dehydrogenases are mitochondrial or cytosolic (as in the present study, since microsomal proteins were not surveyed) considered to take part in the detoxification of oxidant substances like lipid peroxides (Zhang et al. 2010) and the ethanol metabolite acetaldehyde (Yoshida et al. 1989). Aldehyde dehydrogenase has been reported to be significantly down-regulated in *Carcinus maenas* from metal-polluted sites (Montes-Nieto et al. 2010) and in *Mytilus galloprovincialis* (Nasci et al. 2002) due to the antagonist interaction effects of different chemicals. In contrast, this enzyme has also been found to have its activity increased as a result of exposure to environmental the dioxin TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and to the PAH methylcholanthrene (Nakanishi et al. 1979). Regarding HSDs (17β HSDs are a family of known HSDs), although regarded as taking part in steroid hormone metabolism (and for such reason surveyed in the monitoring of endocrine disruptor compounds), they are suspected to have a broad-range of substrates, including xenobiotic by-products, due to their ability to act on carbonyl groups (Odermatt and Nashev 2010). Albertsson et al. (2007), for instance, found that a HSD enzyme (20β HSD) was upregulated in fish exposed to sewage effluents even in the absence of upregulation by the oestrogenic compound 17α ethinylestradiol, suggesting that other compounds were linked to deregulation of the enzyme. Although endocrine disruptors were not surveyed, the present findings may indicate reduced steroid metabolism in laboratory-exposed animals to sediments C_1 and C_2.

To summarize, the proteins that were observed to be differentially regulated as a consequence of exposure to sediments constitute fragments of complex cellular mechanisms potentially triggered by chemical stressors. Whether by 2-DE limitations in spot detection or by failing to identify specific peptides by the MS/MS analyses (mostly due to the yet poor sequencing and annotation of teleost genomes and peptidomes), many key proteins involved in the above discussed processes could not be pin-pointed by the present study. Nevertheless, the results show that a proteomic survey can detect proteins that take part in vital processes of the cell and are deregulated by contaminant action. The most notorious difference between laboratory and in situ bioassays to the contaminated sediments was the reduced induction of most identified proteins in laboratory-exposed fish. It is probable that increased bioavailability, combined with the presence of a mixture of different classes of contaminants, account in part for the differences between laboratory and in situ exposed fish, which sustains the premise that laboratory assays tend to be more conservative. However, in situ assays should permit a more realistic approach determination of the true risks of contamination towards the biota. Caged fish exposed to contaminated sediments appeared to respond more pronouncedly to contamination by organic substances, especially fish exposed to the sediment most contaminated by PAHs and organochlorines. It must also be recognized that deregulation can be an indicator of stress on its own. Even though classic protein biomarkers such as metallothionein or CYP enzymes are often based on the premise that exposure causes induction, it is clear that chemical stress may cause downregulation of many protein responses as a result of general metabolic impairment, at least during early stages of exposure.

Acknowledgments The present research was approved by the Portuguese Science and Technology Foundation (FCT) and POCTI (Programa Operacional Ciência, Tecnologia e Inovação, research project ref. POCTI/AMB 57281/104) and financed by FEDER (European Fund for Regional Development). P. M. Costa was supported by a FCT grant (SFRH/BPD/72564/2010). The authors would also like to thank APSS (Administração dos Portos de Setúbal e Sesimbra, SA); J. Raimundo, V. Branco, R. Cesário and P. Pousão (IPIMAR-INRB) and Carlos A. Fuentes Almagro and Ricardo Fernández Cisnal (Universidad de Córdoba), for their important support.

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