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Teleost fish (*Solea solea*): A novel model for ecotoxicological assay of contaminated sediments

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ABSTRACT

Chemical analysis of sediment is not indicative of the downstream biological effects on aquatic organisms. In this study, the biological effects of sediment were examined using: Teleost fish (*Solea solea*), *Artemia* and rotifers. Although chemicals levels were below the limits permissible by Italian law, *S. solea* juveniles exposed to sediment (0.3%, w/v) for 96 h, revealed significant induction in the expression levels of *HSP70*, *ERα*, *TRα*, *RXRα*, *PPARα*, *PPARβ*, *CYP4501A1* and *CYP3A* mRNAs, suggesting the utility of this species as a novel biosensor. The bio-toxicity of the sediment was further validated by exposing *Artemia* and rotifers to concentrations of elutriate (derived from the sediment) from 10 to 100% (v/v) (with a 50% mortality rate). These results suggest that sediment defined as moderately contaminated, solely on the basis of the chemical profile, may in fact cause harmful effects to aquatic organisms. This study highlights the need for biological approaches in the establishment of sediment toxicity levels.

1. Introduction

In the twentieth century, manufacturing industries have produced and introduced into the environment a plethora of both organic and inorganic contaminants. These contaminants of persistent organic pollutant (POP) include polychlorinated biphenyls (PCB), the pesticide organochlorine pesticides, polycyclic aromatic hydrocarbons (PAH), polychloro dibenzofuran (PCDF), heavy metals (Van der Oost et al., 2003), and phthalates (Carnevali et al., 2010). They are discharged into rivers, lakes and the ocean and in the aquatic environment are spread between water columns (interstitial waters which interface with sediment) and a solid phase (sediment and particulates in suspension) (Luoma, 1983). Chemicals discharged into the environment partitioning between water and sediment (Burton, 2002).

Sediments and aqueous phase, essential for ecosystem functioning, constitute the principal sites of biogeochemical cycles. Additionally they represent an element of fundamental importance in the trophic web (Burton et al., 2001). POPs in different environmental compartments are available for accumulation in aquatic biota (Chapman et al., 2003). POPs represent a potential hazard for ecosystem health, as they can disrupt primary physiological processes in aquatic organisms (Chapman and Mann, 1999). Adverse effects include liver damage, growth retardation, cancer, birth defects, suppression of immunological functions, and reproductive problems (Safe, 2004). As a measure of the magnitude of this problem, 11 billion cubic meters of superficial contaminated sediment have been estimated by the Environmental Protection Agency (EPA) to be present in the first 5 cm of the sediment column. Indeed, this represents a massive index of environmental risk, with potential risk to the entire trophic web, including humans (U.S. EPA, 1997).

Historically, sediment contamination has been assessed by determining the concentration of a single contaminating substance at a given site relative to 'background' or 'reference levels' (U.S. EPA, 1987; Great Lakes Water Quality Board, 1982; Gambrell et al., 1983; Thomas, 1987). More recent studies revealed that only a fraction of contaminants present in sediment were bio-available and exposure levels were much lower than expected (Hamelink et al., 1994; Kraaij, 2001; Celiz et al., 2009; Letcher et al., 2010). Furthermore, as contaminant mixtures may cause synergistic or antagonistic effects, exposure outcomes are not easily defined based on single compound guidelines (de March, 1987; Von Danwitz, 1992; Celiz et al., 2009; Letcher et al., 2010). This has led to newer strategies that evaluate contamination and bioavailability with "*in situ*" chemical

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analysis, and biological assays (Den Besten et al., 2003; Celiz et al., 2009), which permits an assessment of the maximum resiliency point of an organism, from which it is unable to recover from the damage inflicted (Van der Oost et al., 2003).

Although chemical analyses of sediments have proven useful in developing standards for regulating POP exposure in the environment, there is a need for more sensitive assays that can detect the physiological effects of POPs on fish and humans because chronic exposure, especially at low chemical levels, has been shown to disrupt later development in vertebrates (Soto et al., 2008; Grün and Blumberg, 2009; Diamanti-Kandarakis et al., 2009). Recent advances in molecular methods, such as microarrays and quantitative PCR [q-PCR] (Hardiman, 2007; Rouse et al., 2008) have substantially improved the sensitivity for assessing the effects of POPs on animal physiology.

Development of the multi-species microarray was motivated by the need to monitor the endocrine status of turbot, a flatfish that is used as a sentinel for chemical contamination in coastal waters off of southern California (Baker et al., 2009). This microarray can provide a broad measure of the effects of sediment on a variety of endocrine responses in fish. Thus, the microarray included probes for genes encoding receptors for estradiol (*ESR1/ERα*; *ESR2/ERβ*), progesterone (*PR*), testosterone (*AR*), cortisol (*GR*), aldosterone (*MR*), thyroid hormone (*THRA/TRα*;*THRB/TRβ*), retinoids (*RAR*, *RXR*) and vitamin D (*VDR*), and other nuclear receptors: farnesoid X receptor (*FXR*), and pregnane X receptor (*PXR*). Probes for peroxisome proliferators-activated receptor α (*PPARα*), the hydroxysteroid dehydrogenases and the enzymes involved in detoxification (*CYP1A1*, *CYP3A*) were represented (Baker et al., 2009).

We decided to apply these molecular methods to investigate the potential effects of contaminants in sediments on *Solea solea*. We chose *S. solea* as a test fish for two reasons. First, it is an important commercial food. Thus, it is important to prevent exposure to chemicals that affect reproduction and general health of *S. solea*, as well as its suitability as a food for humans. Second, *S. solea* is a sentinel fish for environmental studies because, as a flatfish, it lives in a restricted range. Thus, if there is evidence for exposure of *S. solea* to chemical contaminants, it can be used to determine where the exposure occurred. A problem with using microarrays and PCR for analysis of contaminant effects in *S. solea* is that its genome has not been sequenced. We faced a similar problem in studying turbot, a flatfish that is used as a sentinel for endocrine disruption in coastal waters off of southern California (Baker et al., 2009). Although in principle, the multi-species microarray, which was successfully used for studying endocrine disruption in turbot, could be used for a similar study of *S. solea*, validation of this tool was necessary. Thus, another goal of this project was to determine the versatility of the multi-species microarray for studying endocrine disruption in *S. solea*.

Finally, the last objective of this study was to provide evidence on the validity of the biological approach to establish sediment toxicity using Artemia, rotifers and *S. solea*.

Here we describe the use of the multi-species microarray and a q-PCR panel comprised of the heat shock protein (*HSP70*), the estradiol receptor (*ERα*), peroxisome proliferator-activated receptors alpha and beta (*PPARα*/*β*), Thyroid Receptor alpha (*TRα*), Retinoid X Receprtor (*RXRα*) and Cytochrome P450 3A4 (*CYP3A4*) and 1A1 (*CYP1A1*) to evaluate the effects of an acute 96 h exposure to moderately contaminated sediment on gene expression in *S. solea*. In addition to studying the molecular response in *S. solea* to moderately contaminated sediments, we investigated the effect of sediment derived elutriate on the mortality of two additional species, *Artemia* and rotifers (*Brachionus plicatilis*). Together, these analyses indicate that sediment defined as moderately contaminated, on the basis of the constituent chemical profile, can affect the endocrine response in *S. solea*, and mortality of *Artemia* and rotifers. This approach highlights the advantages of newly developed molecular methods in developing standards for regulating sediment toxicity levels.

Table 1
Primer sequences.

Primers		
Name	Sequence	
ARP	Forward	CTG AAC ATC TCG CCC TTC TC
	Reverse	TAG CCG ATC TGC AGA CAC AC
HSP70	Forward	ACG GAG AGT CGA TT CGA TG
	Reverse	GAA GGA CAT CAG CGA CAA CA
ERα	Forward	ATC TGG AGG TCC ATC CAC TG
	Reverse	GCA AGC AGC ATG TCG AAG AT
PPARα	Forward	TGC AAG GGT TTC TTC AGG AG
	Reverse	GCA GTA CTG GCA CTT GTT GC
PPARβ	Forward	GCT TTC CAG AAG TGC CTG TC
	Reverse	TCT TCA GGT CAG AGC CAC CT
TRα	Forward	GGA AAC AGA AGC GCA AGT TC
	Reverse	TCT TCA CAA GGC AGC TCT GA
RXRα	Forward	GGG CTT CTT CAA GAG GAC AGT
	Reverse	TGC ACC GCT TCT CTC TTC AT
CYP4501A1	Forward	ACC TGT ACA GCT TCC GCT TC
	Reverse	GCA CTG TAG GCC AGC TTT CT
CYP3A	Forward	GAA GCT GTG ATG CAG ATG GA
	Reverse	TGC TGA ACC TTT CAG GTT TG

2. Material and methods

2.1. Chemical and physical analysis

The sediment was sampled in the Northern Adriatic Sea, by ENI according to the international protocol. Chemical analysis of sediment (Table 1) was performed in accordance with the following protocols: USEPA 6020A/98, USEPA 8270C/96, DIN 38414-20, USEPA 1613/94, USEPA 8015B/96, USEPA 8260B/96. Physical analysis of sediment (Table 2) was performed following damp sedimentation and sifting with laser diffraction particle analyzers (LS 200 Bench/Module, 0.4 μm–2 mm).

Chemical analyses of elutriate, to evaluate the concentration in the elutriate of polycyclic aromatic hydrocarbons (PAHs), organochlorinated pesticides (OCPs), polychlorinated biphenyls (PCBs) and C > 12 alkanes were performed using solid phase microextraction (SPME) and gas chromatograph/mass selective detector (GC–MS) (Hewlett Packard, Palo Alto, CA, USA; HP-6890 with HP 5973) techniques.

The presence of the following pollutants (PAHs, OCPs, PCBs and C12 alkanes) in elutriate samples were analyzed using SPME. A 100 μm thickness poly dimethylsiloxane (PDMS) fiber was used as the absorbing material. The fiber was conditioned in the hot injector of the gas chromatograph for 1 h at 250 °C according to the manufacturer's instructions. SPME equilibrium was achieved by directly immersing the fiber into 1.5 ml amber vial capped with PTFE-coated septa, containing 1 ml elutriate at room temperature for 45 min. Magnetic stirring with a 3 mm long Teflon coated stir bar was used to stir the solution at 500 rpm. The fiber was situated off-center in the vial so the sample flew perpendicular to the fiber axis. After the extraction, the fiber was thermally desorbed for 15 min into the glass liner of the GC injector port at 270 °C. Potential carryover was avoided by maintaining the fiber in the injector for an additional time with the injector in the split mode. Reinserting the SPME fiber after the run did not reveal any evident carry over. Moreover, blanks were run periodically during the analysis to

Table 2
Physical characterization of sediment.

Physical characterization (%, w/w)					
Humidity	Sand 1–0.5	Sand 0.5–0.25	Sand 0.25–0.125	Sand 0.125–0.063	Total sand
29.31	0.1	0.7	3.5	38.6	42.9
	Sand 0.063–0.050	Silt 0.05–0.02	Silt 0.02–0.002	Clay <0.002	Total pelite
	17.4	17.1	12.8	9.8	57.1

confirm the absence of contaminants. Quantification of samples was carried out using calibration curves of fortified samples obtained by plotting the relative peak areas versus the concentrations of fortified PCBs or OCPs. Each calibration level was prepared by spiking Milli-Q water at different concentration.

For GC/MS the HP-6890 with HP 5973 (Hewlett Packard, Palo Alto, CA, USA) was used. Separation was performed on an HP 5 MSI column (30 m × 0.25 mm × 0.25 μm film thickness). A HP Chem workstation was used with the GC/MS system. All injections were split and the volume was 2 μl. The flow rate (He) was 0.8 ml/min. The injector temperature was 270 °C. For PAHs, the column temperature program was from 45 °C (1 min) to 180 °C at 25 °C/min, from 180 °C (0 min) to 300 °C at 8 °C/min, then at 300 °C for 12 min. For PCBs, the column temperature program was from 45 °C (1 min) to 190 °C at 8 °C/min, from 190 °C (18 min) to 300 °C at 15 °C/min, then at 300 °C for 10 min. For OCPs, the column temperature program was from 45 °C (1 min) to 190 °C at 8 °C/min, from 190 °C (13 min) to 300 °C at 15 °C/min, then at 300 °C for 10 min. For C12 alkanes, the column temperature program was from 45 °C (5 min) to 220 °C at 10 °C/min, from 220 °C (0 min) to 300 °C at 8 °C/min, then at 300 °C for 10 min. Data were acquired in the electron ionization (EI) mode (70 eV) using the selected ion monitoring (SIM) mode. Before analysis, relevant standards were run to check column performance, peak height and resolution, and the limits of detection. With each set of samples to be analyzed, a solvent blank, a standard mixture and a procedural blank were run in sequence to check for contamination, peak identification and quantification. Compounds were identified mainly by their retention times and mass spectra. Selected samples were analyzed by full-scan GC/MS for confirmation. Detection limits for PAHs, OCPs, PCBs and C12 alkanes were 5 μg/l, 0.05 μg/l, 0.05 μg/l and 5 μg/l, respectively.

2.2. Toxicity test of elutriate with Artemia salina and Brachionus plicatilis

A standard artificial seawater of 35‰ was used for the hatching as well as for the test. After aeration and stabilization for 24 h, the dilution water pH was about of 8.0 ± 0.5 and the oxygen content was about 90% saturation (Vanhaecke et al., 1981). The preparation of sediment elutriates was performed in accordance with the EPA-823-B-01-002 technical manual (EPA, 2001). The elutriate preparation was performed by a mixture of water and sediment (4 parts water to 1 part sediment, v/v) and the mixture stirred for 1 h. The water phase was then separated from the sediment by settling. The final concentrations of 10, 25, 50% (v/v) elutriate in artificial sea water (clean water) and 100% elutriate were used in Artemia and rotifer toxicity tests

The toxicity test of elutriate with *A. salina* was performed as described by Vanhaecke et al. (1981). Artemia cyst hatching was initiated 30 h before the start of the toxicity test. Ten *Artemia nauplii* are transferred with a Pasteur pipette into each dish. The dishes were filled with 10 ml of the respective concentrations of the elutriate, closed, and incubated in darkness at a temperature of 25 ± 1 °C. The control were incubated with artificial seawater. After 24 h the number of dead larvae was counted in each Petri dish. The nauplii are considered dead if no movement of the appendages was observed within 10 sec.

The toxicity test of elutriate with *B. plicatilis* was performed following the standard guide of ASTM (E 1440-91, 2004). Rotifer cysts were induced to hatch in 16 to 22 h by incubating them at 25 °C in standard dilution water. These neonates were then exposed immediately to elutriate concentrations of contaminated sediment plus a control in artificial seawater, in covered dishes. After 24 h, the percent of dead animals in each dishes was recorded. An appropriate statistical method was used to calculate the number of dead rotifer.

2.3. Fish exposure and sampling

Juvenile sole (1.3 ± 0.2 g) obtained from "La Rosa," fish farm (Orbetello, Italy) were acclimatized in tanks for two weeks at 14/10 LD photoperiod and subsequently exposed in 120 l tanks to environmental sediment (0.3% (w/v) moderately contaminated), with constant salinity (30 ppt) and temperature (20 °C). Sole were divided into two experimental groups, (A) (25) fish exposed to uncontaminated sediment (control) and (B) (25) fish exposed to contaminated sediment. The experiment was repeated two times.

Water quality (salinity, pH, O_2, NO_2 and NH_3) tests were performed along all treatment once a day. The fish sampling was carried out at time 0 (T0) and 96 (T96) h following exposure in both the experimental groups. Liver tissue was sampled, flash frozen and stored at −80 °C until RNA extraction was carried out.

2.4. Design of the multi-species endocrine microarray

Development of the multi-species microarray and the probe sets represented has been described in detail elsewhere (Baker et al., 2009). This array was developed initially to monitor the endocrine status of flatfish at different sites in coastal southern California. This tool has global utility facilitating a broad measure of the effects of chemicals on a variety of endocrine responses in fish.

2.5. RNA extraction, fluorescent target labeling and microarray hybridizations

For microarray experimentation, liver tissues were pooled from four individual fish and RNA was extracted as described below. Five hundred nanograms of total RNA were converted into fluorescently labeled Cy 3 or 5 cRNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescent targets were purified to remove unincorporated nucleotides using RNeasy (Qiagen). Absorbance (OD) at 260 nm was used to quantify the cRNA concentrations, and absorbance at 550 nm and 650 nm was used to measure the efficiency of Cy3 and Cy5 dye incorporation. Hybridization was carried out for 18 h at 42 °C in a shaking incubator at 100 rpm. The microarrays were washed with 1× SSC/0.2% SDS for 5 min at room temperature followed by two 5 min washes with 0.1× SSC/0.2% SDS at room temperature. The microarray was rinsed briefly with water and dried by centrifugation at 800 rpm for 5 min. Three biological replicates, each consisting of livers pooled from four fish, were sampled at the start of the experiment (T0), from control fish not exposed to sediment for 96 h (T96 control) and fish exposed to sediment for 96 h (T96 exposed). The T96 control and exposed samples were labeled with Cy 3 and compared to

a T0 reference sample labeled with Cy 3. Data was collected using an Agilent Microarray Scanner and Feature Extraction Software.

2.6. Microarray data analysis

Array data has been deposited in the EBI Array Express Database (accession number E-MTAB-296). Statistical analysis of the microarray experiment involved two steps: normalization of microarray data, and sorting of the genes according to interest as described previously (Baker et al., 2009). We normalized all samples simultaneously using a multiple-*loess* technique described by Šášik et al. (2004).

False discovery rate is calculated using a non-parametric method described below:

The control (C) and treatment (T) samples are paired and a log 2 ratio, log 2(T/C) is calculated for each gene and C–T pair. The genes are ranked according to this ratio. Ranks are integers between 1 and *N*, where *N* is the number of genes. Genes that are truly changed between C and T will have correlated ranks across the replicates, either consistently low or consistently high, depending on the sign of change. This is the distinguishing property between truly regulated genes and genes whose signal is due to random measurement error. The rank-sum statistic, which is the sum of ranks across the replicates, has a well-known distribution when the replicates are statistically independent (Bradley and Gupta, 2002). This is the null distribution. False discovery rate for a gene g is calculated as the ratio of the expected number of genes whose rank-sum statistic is no less extremal than that of g in the null model, and the actual number of genes whose rank-sum statistic is no less extremal than that of g.

2.7. RNA extraction and real time PCR

Total RNA was extracted using TriREAGENT (Sigma) following the recommended protocol and eluted in 50 μl of RNAse-free water. RNA concentrations were determined by Nanodrop (Thermo Fisher Scientific Inc.). 1 μg of RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Invitrogen).

Oligonucleotide primers were designed on the basis of the homology among the sequences present in GenBank.

Each PRC product was extracted by MiniElute PCR purification Kit (Cat n 28004, Qiagen, Germany) and directly sequenced by DMR genomics (Padova, Italy).

A relative quantification of *HSP70*, *ER*α, *TR*α, *RXR*α, *PPAR*α, *PPAR*β, *CYP4501A1* and *CYP3A* mRNA levels was done using the acidic ribosomal protein (ARP) as an internal standard using primers reported in Table 1.

Q-PCR was performed using the SYBR green method on an iQ5 Multicolor real-time PCR detection system (BioRad). Triplicate PCR reactions were carried out for each sample, using the following reaction conditions; 1 μl of diluted (1/10) cDNA, 5 μl of 2*X* concentrated SYBR Green PCR Master Mix (BioRad), containing SYBR Green as fluorescent intercalating agent and 0.2 μM forward primer and reverse primer. Thermo-cycling for all reactions was for 3 min 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at *X* °C (where *X* = 56 °C for *ARP*, *HSP70*, *TR*α, *ER*α, *RXR*α, *PPAR*α and *X* = 60 °C for *CYP3A*) and 20 s at 72 °C. Fluorescence was monitored at the end of every cycle. Dissociation curve analysis was performed and revealed in all cases a single peak. The data obtained were manipulated using the iQ5 optical system software version 2.0.

2.8. Q-PCR data analysis

Data presented in this paper are in the form of mean values (±SD of the mean). The results were examined using a one-way ANOVA followed by a Tukey test, using the statistical software GraphPad Prism version 5.0 (GraphPad Prism Software, Inc., USA). A significance value of $P < 0.001$ was set as the limit of statistical significance.

3. Results

3.1. Chemical/physical analysis

3.1.1. Sediment analysis

The granulometry of the sediment was spread in equal amounts among sand and pelite (Table 2). Chemical characterization revealed the presence of all contaminant classes but at concentrations below the limits allowed by the Italian regulations (D.M., 471/1999, D.M., 367/2003 and D.Lgs 152/2006) (Table 3).

3.1.2. Elutriate analysis

Chemical analysis of the elutriate revealed HPA, PCB, pesticides and heavy metals concentrations (Table 4) at concentrations below the limits allowed by the Italian regulations law.

3.2. Toxicity test with Artemia and B. plicatilis

The test of elutriate toxicity consisted of the following elutriate concentrations: 10, 25, 50 and 100% (v/v). A mortality rate of 50% was achieved for both *A. salina* and *B. plicatilis* with 10% (v/v) final concentration of elutriate (Fig. 1a and b).

3.3. Microarray expression profiling of S. solea exposed to contaminated sediment using an aquatic multi-species endocrine microarray

A heat map of three selected multispecies probe sets; *ERα*, *ERβ* and *PPARα* that were consistently down-regulated (in blue) or up-regulated (in red) in fish exposed to contaminated sediment relative to uncontaminated sediment is presented in Fig. 2. Only multi-species probes that passed strict filtering with a false discovery cut off rate of 0.1 and that exhibited consistent behavior were included in this analysis. Fish exposed to contaminated sediment exhibited increases in the expression of *PPARγ* and *ESR1/ERα* and decreases in the expression of *ESR2/ERβ*.

3.4. Molecular analysis of biomarkers for pollution using Q-PCR

In order to use qRT-PCR to accurately monitor changes in hepatic gene expression, partial sequences of the *S. solea* were performed. Identities of the fragments were confirmed by DNA sequencing and sequence data has been deposited in the NCBI Database. Identity of *ARP* (GeneBank No. GU474637.1), *HSP70* (GeneBank No. GU474638.1), *ERα* (GeneBank No. HO004799), *TR*α (GeneBank No. GU474640.1), *RXRα* (GeneBank No. HO004802), *PPARα* (GeneBank No. HO004800), *PPARβ* (GeneBank No. HO004801), *CYP4501A1* (GeneBank No. HO004797) and *CYP3A* (GeneBank No. HO004798) are showed in Table 5. These partial sole-specific sequences were used for SYBR green quantitative PCR experiments on fish exposed to the moderately contaminated sediment.

3.5. Q-PCR expression analysis

The expression of different genes was evaluated using *ARP* (acidic ribosomal phosphoprotein) as a housekeeping gene.

Quantification of *S. solea HSP70* hepatic gene expression revealed a significant increase in transcript levels following the 96 h (T96) exposure to contaminated sediment, with 3.5-fold changes respect to the controls at both time T0 and T96 (Fig. 3A). *S. solea*

Table 3
Chemical characterization of sediment. ICRAM = Central Institute for Scientific Research Technological and Applied Marine; DM = Ministerial Decree.

Pollutants in the sediment	Concentration (mg/kg)	Law DM 471/99	Law DM 367/03 Tab.2	ICRAM limits
Cadmium	0.11	2–5	0.3	<25–0.20 >25–0.35
Nickel	83.00	120–500	30	<25–32 >25–60
Lead	23.00	100–1000	30	<25–25 >25–37
Copper	18.00	120–600	*N*	<25–15 >25–35
Zinc	49.00	150–1500	*N*	<25–50 >25–100
Mercury	0.25	1–5	0.3	<25–0.20 >25–0.40
Arsenic	10.50	20–50	12	<25–17 >25–23
Vanadium	41.60	90–250	*N*	*N*
Chrome	20.00	150–800	50	<25–50 >25–100
Selenium	0.46	3–15	*N*	*N*
Pyrene	0.05	5–50	*N*	0.153
Chrysene	0.01	5–50	*N*	0.108
Benzo(b)fluoranthene	0.01	0.5–10	0.04	0.04
Benzo(e)pyrene	0.01	*N*	*N*	*N*
Benzo(a)pyrene	0.01	0.1–10	0.03	0.08
Indeno pyrene	0.01	0.1–5	0.07	0.07
Benzo(g,h,i)perylene	0.02	0.1–10	0.055	0.055
Total HPA 471	0.11	10–100	0.2	*N*
Total other HPA	0.04	*N*	*N*	*N*
Total HPA	0.15	10–100	*N*	0.9
Dioxine	1.2E−0.6	1.00E−05–E−04	1.50E−06	*N*
Hydrocarbon C >12	46.00	50–750	*N*	*N*
Hydrocarbon C <12	0.04	10–250	*N*	*N*

Table 4
Chemical analysis of elutriate. E1, E2 and E3 are the 3 replicates of elutriate; DM = Ministerial Decree.

Organic pollutant	Concentration (mg/kg)	Law DM 471/99 (mg/kg)	Law DM 367/03 Tab. 2 (mg/kg)
Pesticides (2,4′-DDE, 4,4′-DDE, 2,4′-DDD, 4,4′-DDD, 2,4′-DDT, 4,4′-DDT)	<0.8	10–100	1.50
PCB (PCB 28, PCB 52, PCB 95, PCB 101, PCB 99, PCB 110, PCB 151, PCB 149, PCB 118, PCB 146, PCB 153, PCB 105, PCB 138, PCB 187, PCB 183, PCB 177, PCB 180, PCB 170)	<0.3	5	0.004
HPA > C12 (n-dodecane, n-tetradecane, n-hexadecane, n-octadecane, n-eicosane n-docosane, n-tetracosane, n-hexacosane, n-octacosane, n-triacontane, n-dotriacontane, n-tetratriacontane, n-hexatriacontane, n-octatriacontane, n-tetracontane)	–	50–750	*N*
HPA (pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, benzo(g,h,i)perylene, dibenzo(a,h)anthracene, indeno(1,2,3-cd) pyrene, dibenzo(a,l)pyrene, dibenzo(a,e)pyrene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene)	<0.2	10–100	*N*

Heavy metal	Concentration (μg/l)	Law DM 471/99 (mg/kg)	Law DM 367/03 Tab.2 (mg/kg)
Nickel	E1 (145,45); E2 (181,38); E3 (375,83)	120–500	30
Lead	E1 (129,08); E2 (114,97); E3 (31,36)	100–1000	30
Chrome	E1 (0,51); E2 (0,73); E3 (4,23)	150–800	50
Mercury	E1 (7,21); E2 (8,16); E3 (11,22)	1–5	0.3

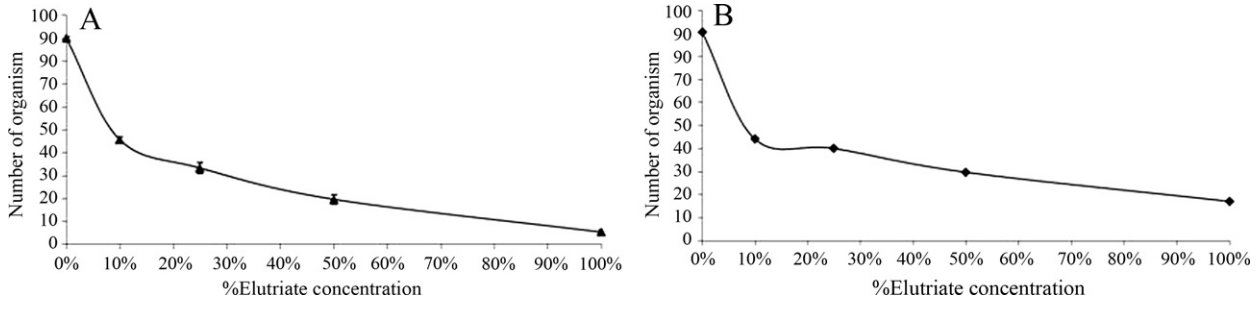

Fig. 1. Elutriate toxicity test. (A) *Brachionus plicatilis*, (B) *Artemia salina*. The lines depict organism survival.

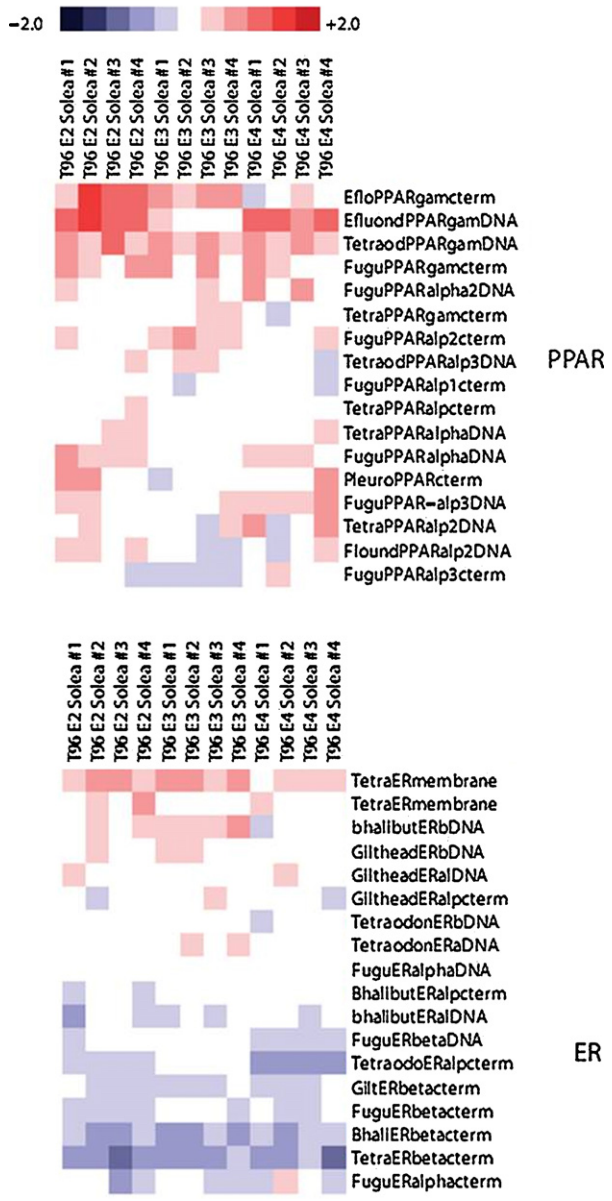

Fig. 2. Multi-species microarray gene expression profiling of sole exposed to contaminated sediment. Gene expression changes were investigated in sole after exposure to uncontaminated and contaminated sediment respectively for a 96 h period. Three biological replicate microarray experiments were performed from control fish exposed to uncontaminated and contaminated sediment. Each sample consisted of livers pooled from four fish. The probe level fold changes observed between clean and contaminated sediment exposed fish for *ER*α, ERα and *PPAR*α probe features are depicted as a heat map for all three experiments. The color range is between −2-fold and +2-fold and preserves qualitative relationships among individual values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 5
Identity analyses of the genes analyzed in *S. solea*.

Gene	Homology (%)	Reference species
ARP	90	*Ictalurus punctatus* (Channel catfish)
Hsp70	99	*Paralichthys olivaceus* (Bastard halibut)
TRα	99	*Solea senegalensis* (Senegalese sole)
ERα	95	*Paralichthys olivaceus* (Bastard halibut)
RXRα	99	*Paralichthys olivaceus* (Bastard halibut)
PPARα	92	*Pleuronectes platessa* (European plaice)
PPARβ	94	*Scophthalmus maximus* (Turbot)
CYP4501A1	95.3	*Pleuronectes platessa* (European plaice)
CYP3A	85	*Platichthys flesus* (European flounder)

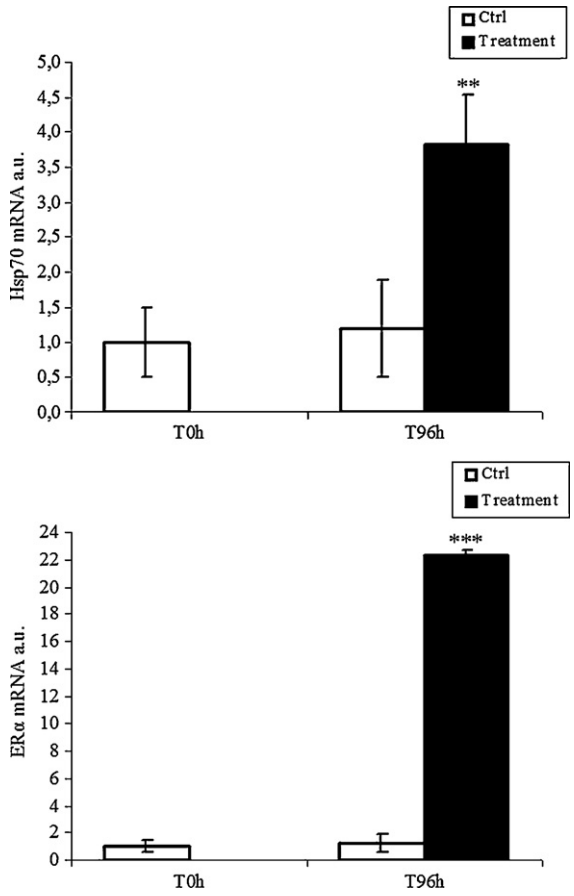

Fig. 3. Stress biomarker assay. Analysis of heat shock protein 70 (*HSP70*) mRNA levels using quantitative PCR. HSP70 mRNA levels in *S. solea* juveniles exposed to uncontaminated sediment (control) in white, and contaminated sediment (treatment) in black ($P < 0.001$) are shown. The data are normalized to the housekeeping gene (*ARP*). Reproductive biomarker assay. Analysis of estrogen receptor α (*ER*α) mRNA levels using quantitative PCR. *ER*α levels in *S. solea* juveniles exposed to uncontaminated sediment (control) in white, and contaminated sediment (treatment) in black ($P < 0.001$) are shown. The data are normalized to the housekeeping gene (*ARP*).

ERα gene expression increased significantly following a 96 h exposure to contaminated sediment with 20-fold changes respect to control(Fig. 3B).

Regarding *PPARα* and *PPARβ* mRNA levels increased significantly following a 96 h exposure to contaminated sediment with 2.5- and 3-fold changes respectively (Fig. 4A and B).

Moreover, *S. solea TRα* and *RXRα* mRNA levels increased significantly following a 96 h exposure to contaminated sediment with 21- and 7-fold changes respectively (Fig. 4C and D).

Finally, *CYP4501A1* and *CIP3A* mRNA levels increased significantly following a 96 h exposure to contaminated sediment with 10- and 12-fold changes respectively respect to the controls groups (Fig. 5A and B).

4. Discussion

The present study examined the biological effects of contaminated marine sediment on the benthonic teleost fish (*S. solea) and on rotifers and Artemia*.

The toxicity test carried out with *Artemia* and rotifers confers with normal Italian contaminant levels (D.M., 471/1999, D.M., 367/2003 and D.Lgs, 152/2006) and was performed under extreme environmental conditions (using an elutriate, representing a concentrate of the pollutants present in the sediment).

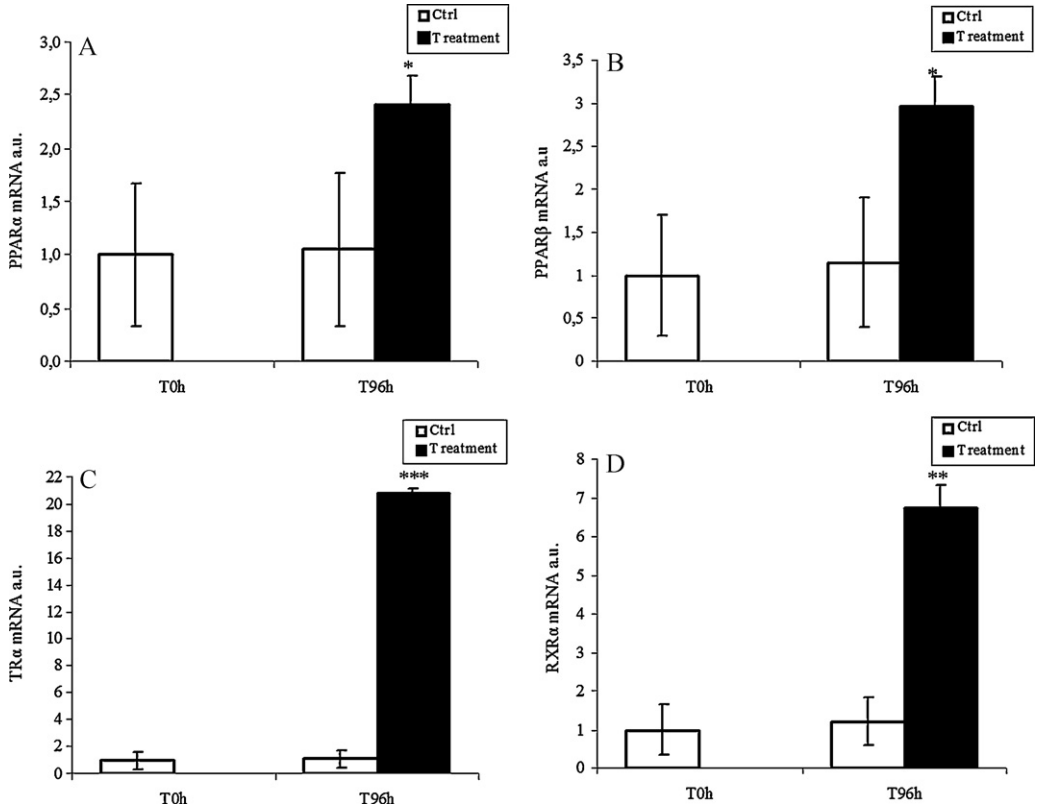

Fig. 4. Metabolic biomarker assay. (Analysis of A and B) Peroxisome proliferators-activated receptor α and β (*PPAR*α and *PPAR*β), (C) thyroid hormone receptor α (*TR*α) and (D) retinoid X receptor α (*RXR*α) mRNA levels using quantitative PCR. Transcript levels in *S. solea* juveniles exposed to uncontaminated sediment (control) in white, and contaminated sediment (treatment) in black ($P < 0.001$) are shown. The data are normalized to the housekeeping gene (*ARP*).

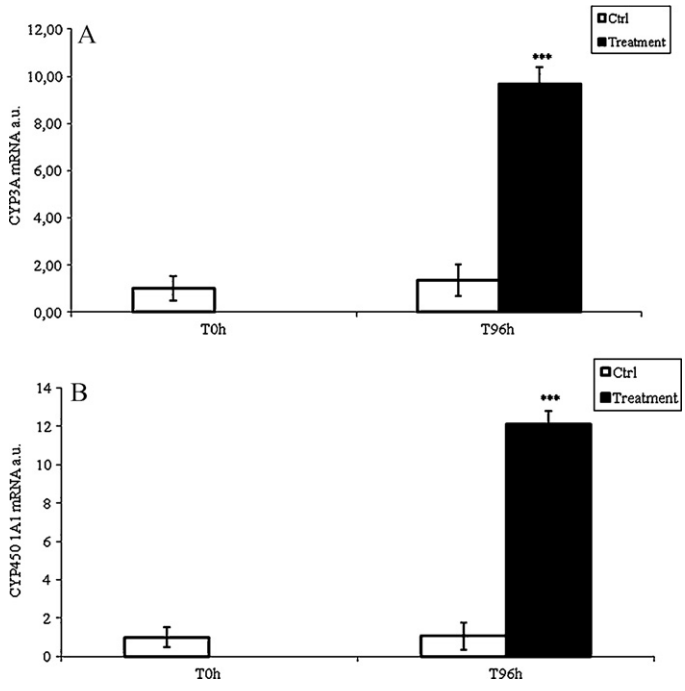

Fig. 5. Detoxification biomarker assay. (Analysis of A) Cytochrome P450 1A1 (*CYP4501A1*) and (B) Cytochrome P450 3A (*CYP3A*). Transcript levels in *S. solea* juveniles exposed to uncontaminated sediment (control) in white, and contaminated sediment (treatment) in black ($P < 0.001$) are shown. These data are normalized to the housekeeping gene (*ARP*).

Despite the low concentration of contaminant in the elutriate (Table 4) we registered a high level of mortality for both species, *Artemia* and rotifer, the lethal effects were evident even at very low final percentages of elutriate dilution. This results suggested us that the biological impact of a mixture of contaminant present in the elutriate despite the chemical concentration of single contaminant may be extremely more detrimental for the life of aquatic organisms such as *Artemia* and rotifers supporting the importance of the biological approach in the management of contaminated system.

Juvenile *S. solea* were exposed for 96 h to contaminated sediment with defined physiochemical properties. As the levels of the primary pollutants present in the sediment did not exceed permissible Italian levels (D.M., 471/1999, D.M., 367/2003 and D.Lgs 152/2006), this sediment was defined as moderately contaminated.

Assessments of sediment quality for the effects of chemical contamination frequently employ a triad of chemical concentration, sediment toxicity, and benthic infaunal community condition data (Long and Chapman, 1985; Chapman, 1990; Chapman and Anderson, 2005; Bay et al., 2007). These approaches are combined as sediments represent a complex matrix and chemical concentration data alone is unable to distinguish between the fraction that is tightly bound to sediment and that which is biologically available.

Recent advances in molecular methods, such as microarrays have substantially improved the sensitivity for assessing sediment quality. The ability to screen a wide range of endocrine responses in organisms from polluted sediment and monitor harmful effluents entering the ecosystem is easily facilitated by microarray and Q-PCR technology. Microarray analysis of alterations in expression patterns – either up or down – in the levels of genes involved in physiological responses to estrogens, androgens, glucocorticoids, thyroid hormone, and detoxification of chemicals provides a powerful tool for obtaining a complete diagnosis of endocrine

disruption in aquatic organisms. A microarray profile of alterations in gene expression associated with a single compound represents a unique signature, which can be used to detect compounds with endocrine disrupting activity in the environment.

The expression of genes involved in the cellular stress response, basal metabolism, reproduction and xenobiotic biotransformation (Iwama et al., 1999; Giguère, 1999; Gu et al., 2000; Wong et al., 2000; Van der Oost et al., 2003; Brown et al., 2004; Woods et al., 2007) were up regulated after the 96 h exposure. All the biomarkers examined in our study can be considered early responses (*ERα*, *TRα*, *CYP4501A1*, *CYP3A*, *HSP70*, *PPARα* and *PPARβ*) to pollution or contamination.

HSP70 is one of the major cellular stress biomarkers, indicating exposure to different classes of organic and inorganic contaminants (Vijayan et al., 1998; Aït-Aïssa et al., 2000; Iwama et al., 1999). An increase in *HSP70* mRNA levels was described following treatment with benzo[a]pyrene, PCB(Grôsvik and Goksôyr, 1996), Nonylphenol (Carnevali and Maradonna, 2003; Maradonna and Carnevali, 2007) and heavy metal (arsenic and cadmium) on *Salmo salar* hepatocyte culture (Grôsvik and Goksôyr, 1996). In the present study a significant increase in *HSP70* gene expression was found indicating the sediment contaminants interfered at a molecular level with the juvenile sole.

In vertebrates TRα is considered the most important nuclear receptor involved in the regulation of basal metabolism (Power et al., 2001). In addition, the complex of TRα and its ligand regulates the expression of target genes involved in development, metamorphosis, metabolism, homeostasis, cellular proliferation and differentiation (Mortensen and Arukwe, 2006). An increase in *TRα* gene expression was previously observed in the liver of the common frog (*Rana temporaria*) exposed to DDE (Mortensen and Arukwe, 2006), and in the rare minnow (*Gobiocypris rarus*) exposed to pesticide amitrole (Li et al., 2009). Considering that many persistent organic pollutants (POPs) and contaminant mixtures can interfere both *in vivo* and *in vitro,* with the thyroid hormone pathway (Janosek et al., 2006), the increase of *TRα* observed in the present study, may be reflective of PCB or organochlorurates present in the sediment. PPARα and PPARβ represent the most important nuclear receptors involved in the regulation of lipidic and glucidic metabolism (Auboeuf et al., 1997; Wu et al., 2001; Wang et al., 2008). PPARα binds many environmental contaminants notably plasticizers and herbicides (Nakajima et al., 2002). Recent studies using human liver cell lines, suggested that activation of PPARα modulates biotransformation processes in the liver through the induction of CYP450 1A1 (Sérée et al., 2004; Fallone et al., 2005). A similar mechanism can be hypothesized with *S. solea* since the increase in *PPARα* was concomitant with an increase in *CYP4501A1* mRNA levels.

RXRα, known also as 9-cis retinoic acid receptor, is a fundamental partner for nuclear receptor transcriptional activity, including TRα and PPARα (Mangelsdorf and Evans, 1995). The pathway mediated by the retinoic acid receptor RXRα is implicated in the biological response to contaminated sediment exposure (Janosek et al., 2006). Nilsson et al. (2000) noted mobilization of retinoic acid in the liver and an increase of retinol in the plasma of mice exposed to tetrachlorodibenzo-p-dioxin (TCDD) (Nilsson et al., 2000).

In the present study the increase of *RXRα* expression was concomitant with increases in *TRα*, *PPARα* and *PPARβ* mRNA levels. RXRα may heterodimerize with these partners to amplify transcriptional activities (Mangelsdorf and Evans, 1995). With regard to ERα, this nuclear receptor binds xenochemical compounds termed "estrogen like molecules" (Blair et al., 1999). These environmental contaminants alter normal functioning of the endocrine and reproductive systems by mimicking or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor isoforms (Sonnenschein and Soto, 1998). In this study, a significant increase in *ERα* transcription was revealed suggesting the presence of estrogen-like substances in the sediment. Estrogenic effects are mediated via an ERE (estrogen response element) in the ER promoter region, that is bound by the ER-estrogen/estrogen like complex and transcribed (Klinge, 2001). An increase in *ER* gene expression in fish exposed to contaminants is generally considered a concise indicator of endocrine disruption.

The interference the contaminated sediment exerts in the fish exposed for 96 h is further demonstrated by the increase of *CYP4501A1* and *CYP3A*, both of which belong to the CYP450 family. These enzymes are involved in biotransformation processes of the primary xenobiotics (HPA, PCB and dioxin) (Goksôyr and Förlin, 1992; Stegeman and Livingstone, 1998; Honkakoski and Negishi, 2000; Coumoul et al., 2002). Numerous studies report increases in *CYP4501A1* gene expression in the liver of teleosts following exposure to organic or environmental contaminants (Nagler and Cyr, 1997; George et al., 2004; Wong et al., 2000; Miller et al., 2004; Fisher et al., 2006; Van Veld et al., 1997). Coumoul et al. (2002) demonstrated that the organochlorides can activate expression of *CYP3A4* in different human cells, including the breast cancer derived mammary cell line (MCF7) and the hepatoma cell line (HepG2).

The higher expression levels of *CYP4501A1* and *CYP3A*, both of which are involved in biotransformation pathways, in exposed juvenile sole may be due to the synergistic effects of contaminants present in the sediment. These results lead us to conclude that the exposure of sole during the most sensitive part of life cycle, namely the juvenile phase, to moderately contaminated sediment induces the up regulation of genes involved in the processes of xenobiotics biotransformation (*CYP4501A1*, *CYP3A*, *RXRα* and *PPARα*), metabolism (*TRα*, *PPARα* and *PPARβ*) and reproductive functions (*ERα*).

The toxic effect of the sediment here analyzed is supported by the results previously obtained in our laboratory on a pelagic species, the sea bream *Sparus aurata* (Ribecco et al., 2011).

This moderately contaminated sediment affected gene expression in this pelagic species as demonstrated by aquatic multispecies microarray analysis and confirmed by q-PCR analysis.

The results of the present study revealed the harmful effects of this sediment in aquatic organisms, in both benthic and pelagic species. This approach demonstrated that integration of both physiochemical analysis and bioassays into toxicity screening provides an excellent framework for elucidating the downstream harmful effects of sediment to aquatic organisms.

However, in the present study, as in the previous one discussed above, only the effect of acute stress was evaluated.

The high magnitude of ERα and TRα (about 20-fold changes) increase, found after 3 days treatment in *S. solea*, lets us suppose a negative role of the sediment here used on both reproduction and metabolism. Although, can be hypothesized that after longer exposure, a reduction of biomarkers levels such as ERα can be found, as observed in other species by a time course exposure with estrogenic compounds (Marlatt et al., 2010). However, the levels of proteins codified by these genes may be still harmful for metabolic and reproductive process. Nevertheless, only by a chronic exposure can be established the real risk of exposure to the sediment here studied. The data here discussed are the first step in this direction.

Moreover, these results suggest that this benthonic experimental model, employed during the juvenile stage of the life cycle, is sensitive to moderately contaminated sediment and represents a suitable model for eco-toxicological studies and environmental monitoring.

In conclusion, chemical sediment classification may not be indicative of the biological effects of sediment on juvenile organisms, *Artemia* and rotifer. The clear physiological response of this experimental model justifies the use of sole juveniles for future environmental risk evaluation. Our *S. solea* model will contribute

to the improved management of contaminated sediment. The genomic biomarkers validated in the present study will have utility in basic biological research, in addition to their use by environmental protection agencies, as a guide for regulatory efforts in industrialized countries to manage sediment contaminant levels.

Additional studies are necessary to establish whether chronic exposure to toxic sediment may affects reproduction of aquatic organism.

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