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Exposure of the gilthead seabream (*Sparus aurata*) to sediments contaminated with heavy metals down-regulates the gene expression of stress biomarkers

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ABSTRACT

Heavy metals incidence in the aquatic environment and its accumulation in fish are under constant review. Gilthead seabream (*Sparus aurata*) specimens were exposed for two weeks to sediments highly concentrated in metals, collected at the Portman Bay (Murcia, Spain). The metals bioaccumulation was tested in liver, muscle and skin. The potential of the sediment exposure to induce variation of the stress biomarkers genes was conducted in liver and skin. Results revealed that sediments were highly contaminated with metals. However, following 2 weeks exposure to the sediments, Cd accumulates only in liver. Interestingly, the expression of the genes *mta, hsp 70* and *hsp 90* were significantly down-regulated in skin. Nevertheless, *cyp1a1* gene was up-regulated only in liver. Results uphold that the stress response magnitude was organ-dependent and the skin was the most responsive tissue to metal stress conditions. These results suggest that skin should be considered as target organ for biomarkers analysis in fishes.

1. Introduction

During many decades, heavy metals issued from mines and related industries were released in marine water. Further, different studies confirmed that these pollutants accumulate along diverse marine food chains [67]. Moreover, fish are an important protein source for humans, for this main reason, if fish gets affected by the pollutants and those fish were consumed, a potential risk for human health could appear [33]. Fish are closely exposed to heavy metals existing in their environment and at present, it is known that the accumulation of heavy metals in fish depends essentially on extrinsic (metal concentration) and intrinsic (fish size and species) factors [40,10,33]. These heavy metals finish by attaining preferential organs or tissues such as liver, muscle or skin [67,40,34].

In fish exposed to heavy metals, at sub-lethal doses, many clear physiological effect were described such as disturbances in respiration [61,69,33], reduction in growth [56], disruption in whole-body or plasma ion regulation [49,6], changes in hematology [74,43,2], enzyme activity [31,30] and other blood parameters, such as glucose, total protein, triglyceride and cortisol that reveal the stress response in fish [13,42]. Indeed, Javed and Usmani [37] reported that significant variation of several biomarkers suggested that fish, exposed to heavy metals, become hypoglycemic, hyperlipidic and hypercholesterolemic. In addition, Martinez et al. [44] suggest that, in continued pollutants exposure, the recovery of some disturbed parameters to control values may occur.

Along with the different disturbances of parameters cited above, different genes, including metallothioneins (MTs), cytochrome P450 1A (CYP1a) and heat-shock proteins (HSPs), are induced by heavy metals. Metallothioneins are low-molecular weight, heat-stable and cysteine-rich proteins involved in the binding and regulation of essential metals such as Cu and Zn, and in the detoxification of non-essential metals such as Cd and Hg [18]. For this reason, the induction of MTs has been frequently used as a

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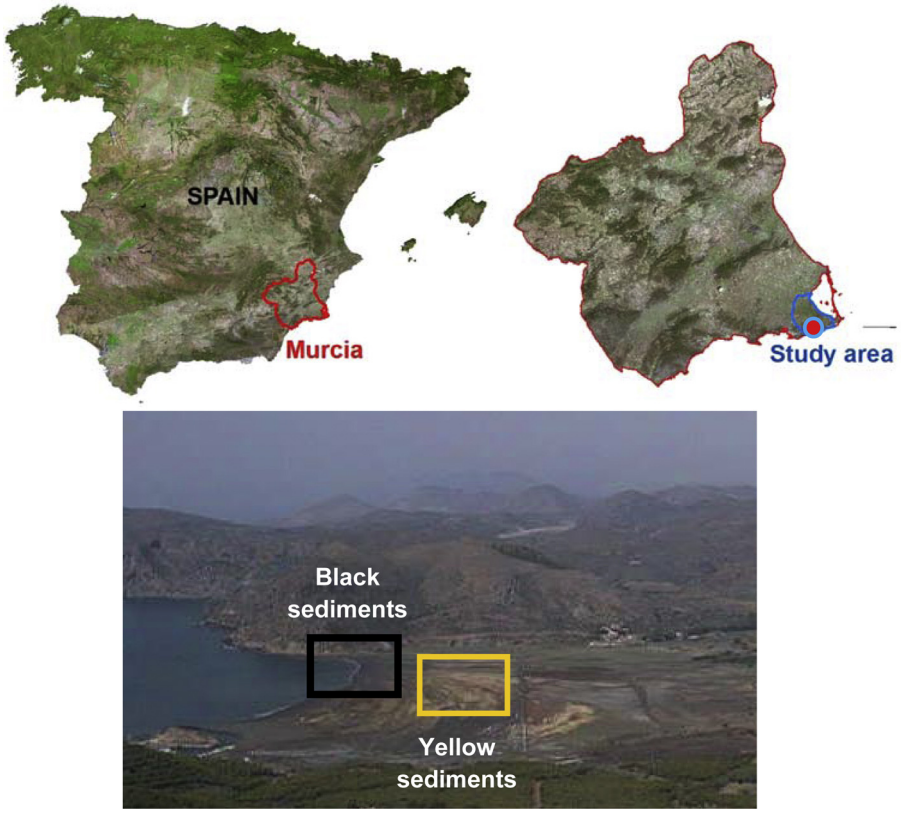

Fig. 1. Photograph of the sampling sites of yellow and black sediments used in the present work from the area of Portman Bay (Murcia, Spain).

biomarker for metal exposure, both under laboratory and field conditions [10]. Concerning *cyp1a*, it is a member of a multigene family of xenobiotic metabolizing enzymes, highly inducible by numerous environmental contaminants and is widely used in biomonitoring studies [50]. Lastly, HSPs, which comprise an evolutionally well conserved protein family, exhibit sophisticated protection mechanisms, and the most conserved HSPs are molecular chaperones that prevent the formation of nonspecific protein aggregates and assist proteins in the acquisition of their native structures [39]. Stress proteins are induced in response to a variety of stress conditions and metabolic insults [70,47,34]. Concomitant damage to DNA was observed with significantly higher in gills (26.5 mm) and liver (20.8 mm) cells of exposed fish compared to control fish [34].

Previous studies agreed that the response of fish to stress conditions depend on the stressor (e.g. temperature, crowding, hypoxia, heavy metals), and also of characteristics of the fish specimens (fish species, age, sex, etc.) [40,1,3]. In the context of the present study, the presence of the heavy metals in sediments will create the stress situation. Usually, studies treating the effect of heavy metals on fish used species seized from polluted sites and these fishes are, in consequence, subject to chronic exposition [73,29,36,33,34]. Other studies handled in laboratory used different concentrations of one or mixture of heavy metals dissolved in water [24,5,25]. In Murcia (Spain) the mining exploration during 33 years, aroused the aboriginal Portman bay as one of the most arid bay in the Mediterranean Sea. This is the first study intending to look over the potential biological restoration of the bay.

In the present study, we aimed to evaluate the impact of heavy metals polluted sediments on gilthead seabream (*Sparus aurata*) as this teleost is more than half of the total marine fish production in Spain. To do this, specimens of gilthead seabream were subject to acute exposition during two weeks. The effect of this short acute exposition was investigated firstly through the bioaccumulation of three toxic heavy metals (Cd, Pb and As) in the liver as main organ implicated in the metabolism of xenobiotics and in two edible organs (muscle and skin). Secondly, we analyzed the behavior of the main genes, inducible under stress conditions, in liver which is known as the main organ of metabolism of toxic pollutants and in the skin that represents the first defense line of fish. Additionally, potential risks for fish health and consumers as well as the possible role of gilthead seabream in the restoration of polluted environments will be discussed.

2. Materials and methods

2.1. Sediments

The sediments selected for this work were taken from Portman Bay (South-Eastern of Spain), one of the most polluted area from the Mediterranean Sea as a consequence of a mining industry some years ago. In fact, during 1958–1991, this bay was the destiny of 67 millions of Tm of residues that shifted the shoreline 800 m to a depth of 24 m [45]. Sterile discharges carrying potential toxic elements (PTE) as As, Pb, Cd and Zn, and released in marine environment for about 33 years, were collected. The samples of marine sediments were not homogeneous and can easily be differentiated in two very different types. First, sediment grain size sand sediment size, dark color (black sediment) that comes from material washed into the sea and dragged to the beach by the action of marine dynamics. The second sediment, yellowish brown (yellow sediment), very fine grain, which would be equivalent to the original sediment spill, which has not had a chance to wash in the sea poured directly on the beach. The physical and chemical characteristics of both pellets are summarized in Tables 1 and 2 [46]. For the present work, going from the coast to the beach, two types of sediments, yellow and black sediments, were collected at 0–15 cm

Table 1
Resume of physical characteristics of sediments.

	Texture%		
Sediment	<5 μm	<63 μm	<250 μm
Yellow Sediment	25.0	34.4	100.0
Black Sediment	0.4	3.1	15.9

depth, air-dried and sieved through a 2 mm screen (Fig. 1). Since the pH of pure water is equal to 7 ($[H^+] = [OH^-] = 10^{-7}$ M), in this study, the pH of the sediments was determined in a 1:5 (w/v) suspension of sediment in pure water [45].

2.2. Experiment design and fish rearing conditions

Four aquaria of 30 l were disposed: The first aquarium was filled with seawater (control) (pH 7.2), the second contained 8 kg of black sediment, the third one 8 kg of yellow sediment and the fourth aquarium 8 kg of mixed sediment (70% of black sediment and 30% of yellow sediment). The three aquaria containing sediments were filled with the same seawater used in control aquaria. Afterwards, aquaria re-circulating systems remained running for one week without fishes. The water was maintained at 20 ± 2 °C with a flow rate of 75 l h^{-1} and 28‰ salinity and the photoperiod were of 12 h light: 12 h dark. The pH of the water in aquaria was determined after 7 days and samples of 100 ml of water and 100 g of sediments were used to determine metal concentrations.

Forty eight specimens (11.91 ± 3.3 g body weight and 8.94 ± 1.45 cm body-length) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.) were obtained from the local farm and they were allowed to acclimatise (in aquaria containing only seawater) for 15 days before the start of the experimental trial. Afterwards, fish were distributed in the four aquaria (12 fish per aquarium). Fish were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. Prior to sampling, fish were starved for 24 h and sacrificed by an overdose of MS222 (Sandoz, 100 mg ml^{-1} water) [21]. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.3. Fish sample collection

Fish were sampled after 15 days of exposure. Whole fishes were weighted and dissected under sterile conditions. Fragments of liver, skin and muscle were took and stored at −80 °C for later determination of heavy metals accumulation. Liver and skin fragments were also sampled for RNA extraction and gene expression evaluation.

2.4. Metals quantification

2.4.1. In sediments and water

Sediment samples of 5 g (air dried for one week) were first ground to a fine powder using a zirconium ball mill. Aliquots of 0.1 g of sediment samples were placed in Teflon vessels, and 5 ml of concentrated hydrofluoric (HF) acid solution, 200 μl of concentrated nitric acid (HNO_3, Sigma) acid solution and 5 ml of distilled water were added. When digestion in a conventional microwave oven was complete (15 min at 1000 W in a Milestone ETHOS PLUS microwave oven), the solutions were transferred to a volumetric flasks and brought to 50 ml. Heavy metal content was determined in each one of the extracts of a 1:5 (w/v) sediment-water mixture once they were filtered, representing the soluble fraction. The Zn and Fe content were determined by flame atomic absorption spectrometry (FAAS). The Pb, Cd and Cu content was determined by electrothermal atomisation atomic absorption spectrometry (ETAAS) (CONTRA 700). The As content was measured by atomic fluorescence spectrometry using an automated continuous flow hydride generation spectrometer (PSA Millenium Merlin 10055 for arsenic). The reliability of the results was verified by analyzing standard reference materials.

The quantification of metals in water samples handled from all aquaria was determined following the same protocol used for the soluble fraction issued from the mixture sediments-water cited above.

2.4.2. In fish

Liver, skin and muscle samples, previously stored at −80 °C, were lyophilized and 100–200 mg of the resulting powder were placed in Teflon vessels with 3 ml of water, 2 ml of concentrated H_2O_2 and 5 ml of concentrated HNO_3 acid solution. The digestion of the samples was carried out using a Milestone ETHOS Plus Microwave system operating with a standard program (85, 200, 210 and 0 °C for 2, 8, 10 and 20 min, respectively). Finally, 50 μl of the solutions were used to determine the metals concentration using atomic fluorescence spectrometry with an automated continuous flow hydride generation (HG-AFS) spectrometer (PSA Millenium Excalibur 10055). Quality control of the analytical was used of reference materials: DOLT-2 Dogfish liver, DORM-2 Dogfish muscle. The recovery obtained with the reference materials was above 93% in all cases. Data are presented as μg of metal per kg dry-weight tissue [25].

2.5. Real-time PCR

Total RNA was extracted from 0.5 g of seabream skin and liver tissues using TRIzol Reagent [16]. It was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8–2.0. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA)

Table 2
Statistical resume of chemical characteristics of sediments.

TOTAL CONCENTRATION						
Sediment	Statistical analysis	Fe (%)	Pb (mg kg^{-1})	Cd (mg kg^{-1})	Cu (mg kg^{-1})	As (mg kg^{-1})
Yellow Sediment	Range	14.30–40.91	1373–7676	10.2–35.5	19–308	319–6620
	Average ± SD	29.07 ± 6.51	4407.4 ± 1910.2	16.2 ± 7.6	60.1 ± 61.8	1249.1 ± 1411.0
Black Sediment	Range	23.11–60.25	1580–4701	8.9–28.3	37–95	248–814
	Average ± SD	40.13 ± 15.18	2812.8 ± 782.1	13.3 ± 4.9	64.1 ± 18.4	493.5 ± 173.0
Water SOLUBLE CONCENTRATION						
Sediment	Statistical analysis	Fe (mg kg^{-1})	Pb (mg kg^{-1})	Cd (mg kg^{-1})	Cu (mg kg^{-1})	As (mg kg^{-1})
Yellow	Range	50–150	0.04–0.08	0.1–2.4	1.3–1.9	2.4–18.8
Sediment	Average ± SD	78 ± 30	0.10 ± 0.02	0.9 ± 1.2	1.7 ± 0.3	11.3 ± 8.3
Black	Range	< lc-2	0.01–0.01	0.003–0.02	0.004–0.03	0.002–0.004
Sediment	Average ± SD	1 ± 0.3	0.01 ± 0.005	0.01 ± 0.01	0.01 ± 0.01	0.002 ± 0.001

Table 3
Primers used for real-time PCR.

Gene name	Gene abbreviation	GenBank number	Primer sequences (5′ → 3′)
Elongation factor 1α	*ef1a*	AF184170	CTGTCAAGGAAATCCGTCGTTGACCTGAGCGTTGAAGTTG
Metallothionein A	*mta*	X97276	ACAAACTGCTCCTGCACCTCCAGCTAGTGTCGCACGTCTT
Heat-shock Protein 70	*hsp70*	EU805481	AATGTTCTGCGCATCATCAAGCCTCCACCAAGATCAAAGA
Heat-shock Protein 90	*hsp90*	DQ524994	GGAGCTGAACAAGACCAAGCAGGTGATCCTCCCAGTCGTT
Cytochrome P450, family 1, subfamily A, polypeptide 1	*cyp1a1*	AF011223	GCATCAACGACCGCTTCAACGCCCTACAACCTTCTCATCCGACATCTGG

Table 4a
Concentration of metals in sediments of the four experiment trial. Four aquaria of 30 l were disposed: The first was filled with seawater (control), the second contained 8 kg of black sediment, the third one 8 kg of yellow sediment and the fourth aquarium 8 kg of mixed sediment (70% of black sediment and 30% of yellow sediment). The three aquaria containing sediments were filled with the same seawater used in control aquaria. Metals in sediments from each aquarium were analyzed.

Aquaria content	Cd ($mg\,kg^{-1}$)	Cu ($mg\,kg^{-1}$)	Pb ($mg\,kg^{-1}$)	Fe (%)	Zn ($mg\,kg^{-1}$)	As ($mg\,kg^{-1}$)
Only water	–	–	–	–	–	–
Black Sediment	6.7 ± 0.33	30.2 ± 0.45	143.00 ± 10	34.2 ± 0.34	6875 ± 206	100.0 ± 4
Yellow Sediment	8.8 ± 0.44	32.6 ± 0.48	508.0 ± 35	32.78 ± 0.32	7763.0 ± 232	300.0 ± 12
Mixed sediment	8.1 ± 0.4	31.0 ± 0.46	398.0 ± 27	33.1 ± 0.33	7490 ± 224	160.1 ± 6.4
Quantification limit ($mg\,kg^{-1}$)	0.03	0.1	0.3	0.01	0.5	0.03

Table 4b
Concentration of metals in water of the four experiment trial. Four aquaria of 30 l were disposed: The first was filled with seawater (control), the second contained 8 kg of black sediment, the third one 8 kg of yellow sediment and the fourth aquarium 8 kg of mixed sediment (70% of black sediment and 30% of yellow sediment). The three aquaria containing sediments were filled with the same seawater used in control aquaria. Metals in water from each aquarium were analyzed after 15 days.

Aquaria content	Cd ($\mu g\,L^{-1}$)	Cu ($\mu g\,L^{-1}$)	Pb ($\mu g\,L^{-1}$)	Fe (%)	Zn ($mg\,L^{-1}$)	As ($\mu g\,L^{-1}$)
Only water	<ld	<ld	<ld	<ld	<ld	<ld
Black Sediment	<ld	<ld	<ld	<ld	<ld	<ld
Yellow Sediment	0.4 ± 0.1	<ld	<ld	<ld	148 ± 3.0	1.5 ± 0.1
Mixed sediment	<ld	<ld	<ld	<ld	<ld	0.4 ± 0.1
Detection limit (ld)	0.1	0.5	1	0.3	0.02	0.1

was synthesized from 1 μg of total RNA using the SuperScript III reverse transcriptase (Invitrogen) with an oligo-dT18 primer. A real-time PCR was performed for the expression of the selected genes (mta, cyp 1A1, hsp 70 and hsp 90). The real-time PCR was implemented by an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μl of 2 x SYBR Green supermix, 5 μl of primers (0.6 μM each) and 5 μl of cDNA template) were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1*a) RNA content in each sample. The reaction efficiency of *ef1a* was 113%, analyzed by serial five-fold dilutions, calculating the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA. Gene names follow the accepted nomenclature for zebrafish (http://zfin.org/). The primers used in the present study are shown in Table 3. In all cases, each PCR was performed with triplicate samples.

2.6. Statistical analysis

All the bioassays were done in triplicate, and the mean ± standard error (SE) for each group was calculated. Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19 and differences were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. pH in sediments and water

The yellow sediments (placed in the coast) have a pH of 1.2 while the black sediment (placed more far from the beach) have a pH of 7.6. The final pH of the water in aquaria containing black, yellow and mixed sediments was 7.6 (±0.1), 2.8 (±0.2) and 6.9 (±0.1), respectively. In the experiment handled with yellow sediment all seabream specimens died after less than 24 h of exposure, in consequence only three different experimental groups could be considered in the trial.

3.2. Metals quantification in sediments and water

The chemical analysis of sediments showed that they are concentrated in different metals. Black, yellow and mixed sediments contained almost the same quantity of Cd, Cu, Fe, and Zn. However, we noted that yellow sediment were 3.5 fold concentrated in Pb ($508\,mg\,kg^{-1}$) than black sediment ($143\,mg\,kg^{-1}$). In contrast yellow sediment was 3 fold concentrated in As ($300\,mg\,kg^{-1}$) compared to the black sediment ($100\,mg\,kg^{-1}$) (Table 4a).

The quantity and nature of heavy metal present in the water of each one of the aquaria used in the present work depended on the type of sediment used. Samples taken from aquaria contained only water (control) or black sediment did not contain any metal traces (Table 4b). However, samples of water from aquaria contained yellow sediment had $0.4\,\mu g\,l^{-1}$ of Cd, $1.5\,\mu g\,l^{-1}$ of As and $148\,mg\,l^{-1}$ of

Table 5
Non-essential heavy metals (Cd, As, Pb) concentrations in liver, muscle and skin of gilthead seabream (*Sparus aurata* L.) confined during 15 days in aquaria containing: W, only water (control); B, black sediment; Y, yellow sediment; M, mixed sediment. <Lc: inferior to quantification limit. NR, not realized. Lc of Cd: 0.03 mg kg^{-1}, Lc of As: 0.03 mg kg^{-1}, Lc of Pb: 0.3 mg kg^{-1}.

Heavy metal	Liver				Muscle				Skin			
	W	B	Y	M	W	B	Y	M	W	B	Y	M
Cd (mg kg^{-1})	<Lc	<Lc	NR	0.5	<Lc	<Lc	NR	<Lc	<Lc	<Lc	NR	<Lc
As (mg kg^{-1})	<Lc	<Lc	NR	<Lc	<Lc	<Lc	NR	<Lc	<Lc	<Lc	NR	<Lc
Pb (mg kg^{-1})	<Lc	<Lc	NR	<Lc	<Lc	<Lc	NR	<Lc	<Lc	<Lc	NR	<Lc

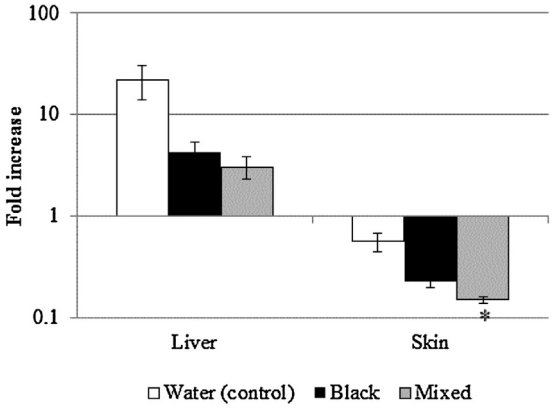

Fig. 2. Expression of metallothionein (*mta*) gene determined by real-time PCR in liver and skin of gilthead seabream specimens after 15 days of exposure to heavy metals polluted sediments. All values represent the mean ± S.D. (n = 6) fold increase relative to control. Asterisks denote significant differences between unexposed (control) and polluted sediments exposed groups ($P \leq 0.05$).

Zn. Furthermore, in the water sampled from the aquaria contained mixed sediments a concentration of 0.4 μg l^{-1} of As was detected.

3.3. Metals bioaccumulation in liver, muscle and skin

The concentrations of Cd, Pb and As detected in liver, muscle and skin of gilthead seabream reared for 15 days in aquaria contained different type of polluted sediments is resumed in Table 5. The concentrations of Cd, Pb and As in muscle and skin of fish specimens reared in water (control) and aquaria contained black sediment were always below the quantification limit (cl; <0.1 μg kg dry-weight^{-1}). Furthermore, Cd was detected in the liver of all seabream specimens reared in aquaria containing mixed sediments (0.5 mg kg dry weight^{-1}) (Table 5).

3.4. Stress biomarkers gene expression

It is well documented that the liver is the main organ implicated in the metabolism of xenobiotic agents. As well, the skin is the external semipermeable tissue allowing fish to exchange with their environment and it represents their first barrier of defense. Taking in account the crucial roles of liver and skin, we analyzed in these organs the expression of *mta*, *hsp70*, *hsp90* and *cyp1a1* genes known to be inducible in stress conditions.

3.5. Evaluation of the mta gene expression

The expression of the *mta* gene in liver and skin of seabream reared in the different aquaria is presented in Fig. 2. After 15 days of exposure, the expression of the *mta* gene of liver from seabream specimens reared in aquaria containing black or mixed sediments exhibit a slight not significant down-regulation compared to specimens reared in control (only water) aquarium. Similarly, the expression of the *mta* gene in the skin of specimens reared in aquaria containing black or mixed sediments was also down-regulated although only in a significant extent in specimens reared in aquaria with mixed sediments (Fig. 2).

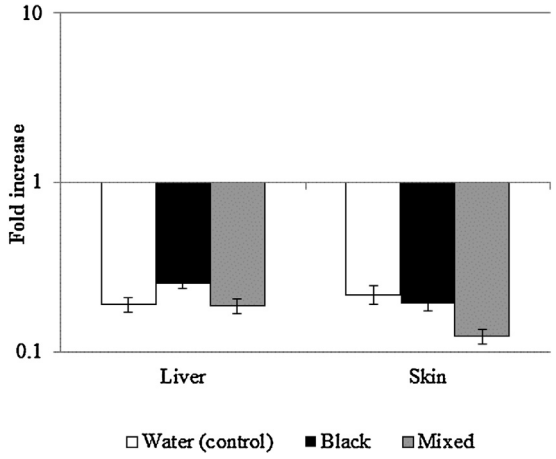

Fig. 3. Expression of heat-shock protein 70 (*hsp70*) gene determined by real-time PCR in liver and skin of gilthead seabream specimens after 15 days of exposure to heavy metals polluted sediments. All values represent the mean ± S.D. (n = 6) fold increase relative to control.

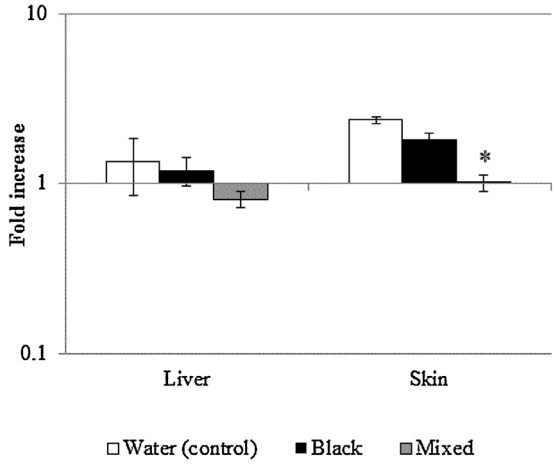

Fig. 4. Expression of heat-shock protein 90 (*hsp90*) gene determined by real-time PCR in liver and skin of gilthead seabream specimens after 15 days of exposure to heavy metals polluted sediments. All values represent the mean ± S.D. (n = 6) fold increase relative to control. Asterisks denote significant differences between unexposed (control) and polluted sediments exposed groups ($P \leq 0.05$).

3.6. Evaluation of Heat-Shock proteins gene expression

The expression of the genes *hsp70* and *hsp90* in liver of seabream specimens reared in aquaria containing black or mixed sediment did not show any significant difference compared to the expression found in liver from control group (aquaria containing only water) (Figs. 3 and 4). However, the expression of *hsp* genes in skin of specimens reared in aquaria containing black sediments were slightly down regulated but did not reach significant levels compared to specimens reared in control aquarium (Figs. 3 and 4). Similarly, the expression of such genes in the skin of specimens reared in aquarium containing mixed sediments was down-regulated, compared to the values obtained in control fish, although the differences were only statistically significant for the *hsp90* gene (Figs. 3 and 4).

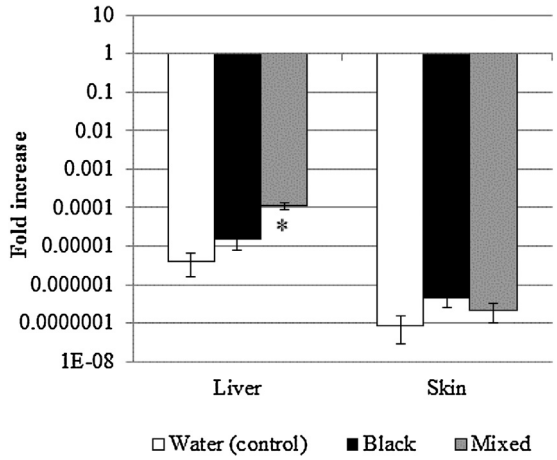

Fig. 5. Expression of gene cytochrome P450 1A (cyp1a1) determined by real-time PCR in liver and skin of gilthead seabream specimens after 15 days of exposure to heavy metals polluted sediments. All values represent the mean ± S.D. (n = 6) fold increase relative to control. Asterisks denote significant differences between unexposed and polluted sediments exposed groups ($P \leq 0.05$).

3.7. Evaluation of cytochrome P450 gene expression

The expression of *cyp1a1* gene in liver of all gilthead seabream specimens reared in aquaria containing black or mixed sediments was increased being the increments statistically significant (twenty eight up-regulation fold) in those fish maintained in aquaria with mixed sediments, compared to the values obtained in liver from control fish (aquaria with only water) (Fig. 5). Expression of *cyp1a1* gene in skin from specimens reared in aquaria containing black or mixed sediments showed no statistically significant variations with respect to control (Fig. 5).

4. Discussion

Due to the release of effluents issued from mining activity during more than three decades, the natural sediments of Portman Bay were highly polluted by essential and non-essential metals. In consequence, these sediments were disastrous for both phytoplankton and zooplankton species. Furthermore, sediments are an important habitat component in aquatic ecosystems, as they provide a substrate or support medium for aquatic organisms. In addition, sediments are biogeochemically active, in that they may transport organic or inorganic substances attached to their surfaces, and because they provide a large surface for abundance of microbes [12]. In Portman bay, several assays for deployment of polluted sediments and restoration of the bay started since 1990 [53]. But the biological aspect was usually neglected. Generally, in fish, under natural exposure conditions, prediction of toxic effects based on environmental or tissues concentrations remains difficult although many studies have examined the relationship between metal exposure, accumulation and toxicity under laboratory conditions [3]. In the present study we intended to reproduce environmental conditions similar to that found in Portman bay and to analyze the effect of acute exposure of gilthead seabream to different highly polluted sediments sampled from different emplacement of such bay. To do this, gilthead seabream were directly exposed to three different polluted sediments sampled from Portman bay: yellow, black and mixed (70% of black and 30% of yellow) sediments. As it was expected, all gilthead seabream specimens reared in aquaria with yellow sediment died in less than 24 h, which can be explained by the very acid pH of the water (pH 2.8) in this aquarium. However, seabream specimens reared in aquaria with black or mixed sediments did reveal neither mortality nor visible negative effects.

Analysis of sediments (yellow, black and mixed) sampled from aquaria after 15 days of trial revealed that they were highly concentrated in all analyzed metals (Cd, Cu, Pb, Fe, Zn, As). In fact, the concentration of Cd ranged between 8.1 mg kg^{-1} and 8.8 mg kg^{-1} and the concentration of Pb ranged between 143 mg kg^{-1} and 508 mg kg^{-1}. However, for the As the concentration oscillated between 100 mg kg^{-1} and 300 mg kg^{-1}. In a recent study, concentrations of As and Cd equal to 37.5 μg l^{-1} and 0.57 μg l^{-1} respectively, were sufficient to be considered sub-lethal for catfish (*Clarias batrachus* L.) and rainbow trout [63,30]. Chemical analysis of water sampled from control aquarium (containing only seawater) and water sampled from aquaria containing black sediments, showed that the concentration of analyzed metals remain lower than the quantification limit. Nevertheless, the analysis of water sampled from aquaria containing yellow and mixed sediments demonstrated the presence of low concentrations of As in these aquaria (1.5 μg l^{-1} and 0.4 μg l^{-1}, respectively). The concentrations of As are usually within the range of 1–2 μg l^{-1} of non-contaminated seawater Cornelis [17]. Many studies showed that As disturb metabolism of cells, causes immunosuppression and increase susceptibility to infection [71,60], induce oxidative stress [11], apoptosis of fish hepatocytes [20] and liver inflammation [52].

Essential metals (Zn, Cu, Fe) and non-essential metals (Cd, As, Pb) have been demonstrated to accumulate along the trophic chain in marine environments [67,64]. Non-essential metals are not known to play any metabolic function although, as a consequence to their bioaccumulation in fish, these metals can be toxic for humans, even at very low concentrations [9]. In the present study, the concentration of Cd, Pb and As in muscle and skin were always below the quantification limit (cl; <0.1 μg kg dry-weight^{-1}) after 15 days of exposure in all experiments. However, Cd was detected in the liver of all seabream specimens reared in the aquarium containing mixed sediments (0.5 mg kg dry weight^{-1}). In fact, it has been suggested that Cd is absorbed through passive diffusion or carrier mediated transport over the gills or absorbed by endocytosis through intestine [54]. Cadmium ions enter the chloride cells in the gills through calcium channels [51]. Once enter in the cells the metal is made available for the interaction with cytoplasmatic components such as enzymes (causing toxic effects) and metallothioneine (probably being detoxified) [54].

The accumulation pattern of contaminants in fish, and in other aquatic organisms, depends on their nutrition uptake, their position along the trophic chain, as well as uptake and elimination rates [26,62]. For gilthead seabream, the accumulation of heavy metals in liver, in real-world polluted aquatic environment, could probably increase. In fact, gilthead seabream is well known as carnivorous fish and it is placed in higher level than zooplankton and phytoplankton in the trophic chain [57]. It is known that liver represent an important storage of metals in animals [41,58,38,59]. After exposure to heavy metals, the hepatic cells started progressively to loose typical cellular organization including necrosis, peripheral displacement of nuclei, indistinguishable cell membrane [55,65,19]. Javed et al. [33]reported that the protein levels increased in the liver of fish exposed to heavy metals. Literally, they suggested that this increases could also be due to the effort of liver to repair their damaged tissues as a result low levels were reported in serum despite synthesis. Thus, the study of the liver is more often recommended as the environmental indicator tissue of pollution than other fish organs [59].

In fish, the skin is considered as a natural, physical, biochemical, dynamic, and semipermeable barrier that enables the exchange of nutrients, water, hormones [22,8]. Admitting this substantially function of skin, in our study, metals concentrations were always below the quantification limit (cl; <0.1 μg kg dry-weight^{-1}) after 15

days of exposure. These results agree with the founding of Handy [27] who described reduced Cd bioaccumulation in skin compared to liver, kidney and gills. However, the analysis of metals accumulation in muscle, after 15 days of exposure, revealed that they are below the quantification limit, which is in accordance to previous data indicating that Cd concentration in seabream muscle, after 30 days of exposure, was very low or undetectable [25]. These levels of heavy metals in skin (and also in muscle) suggest that the principal metals could entry to fish across other organs such as gut or gills. Nevertheless, the accumulation of metals in the fish depends on fish species and studied organ. Javed et al. [33] demonstrated that, in *Channa punctatus* specimens exposed to heavy metals, the Zn had the highest accumulation levels in the liver. Other investigators have also reported the highest accumulation of Zn in the gills of fish such as *Labeo umbratus* [14], *C. punctatus* [68], *Gambusia Holbrooki* [23], *Cyprinus carpio* [32], and *Clarias gariepinus* [35]. Taking in account, for example, the maximum permitted levels of Cd into fish (0.5 μg Cd g dry-weight^{-1}; European Commission Regulation N° 1881/2006), our funding could reassure human consumer and aquaculture farms for intake of gilthead seabream reared in heavy metals polluted environments, for at least 15 days.

Considering the great number of factors interfering in the metals bioaccumulation and metabolism, as well as the inter-specific and intra-specific sensitivity variation of fish face to these xenobiotic, recently, toxicologists are more oriented to reveal heavy metals accumulation using the variation of the expression of some gene considered as biomarkers. In fact, biomarkers represent early indications of biological effects rather than the chemical biomonitoring of polluted sites. The relevance of some genes as biomarkers in fish was related to their ability to signal sub-lethal concentrations of metals ions as well as their biological significance [15]. A battery of biomarkers consisting of specific stress indices should be utilized. In this context, we used an association of genes, known in literature to be implicated in the metabolism of xenobiotics, to reveal potential influences of heavy metals pollution on seabream. In fact, *mta*, *hsp 70, hsp 90* and *cyp1a1* are the most common genes induced in stress conditions and used as biomarkers. In the present study, after 15 days of exposure, the expression of the *mta* gene in gilthead seabream was down-regulated in both liver and skin. This down-regulation reached a significant level in the skin of specimens reared with mixed sediments. These results corroborate with the finding of Bargelloni et al. [7] who concluded that *mta* gene is expressed differentially in different tissue. Moreover, it has been argued that the toxicity of heavy metals such as Cd will only occur when the binding capacity of metallothionein is exceeded and the excess heavy metal is free to bind some other proteins in the cell [54]. On the other side, it has been argued that the kinetics of metal uptake and metallothionein synthesis are both to be taken into account, whereby the pathological effects would appear when the rate of metal uptake exceeds the rate of metallothionein synthesis [48]. Javed et al. [33] reported that the high accumulation of Zn in gills might be due to Zn metallothionein induction, since their production increases due to the elevated levels of heavy metals (Zn, Cu) which bind to the metals in order to detoxify them; in doing so, they concentrate and regulate metals in the organs. Other studies suggested that the down-regulation of some biomarkers genes is due to some factors acting on mRNA stability and translation. In fact, the translation of ferritin mRNA is down-regulated by an RNA-binding protein which interacts with a hairpin motif in the 5′-UTR (UnTranslated Region) [28].

In the present study, we noticed a slight down-regulation of *hsp70* gene in the skin of specimens reared in black and mixed sediments but still not reached significant extent. However, a significant variation of *hsp90* gene in skin of seabream reared in mixed sediments. These variations of *hsp70* and *hsp90* genes detected in the skin of seabream could be explicated by the role of skin as first barrier protecting fish. In fact, when cells are subjected to environmental changes, HSPs are induced and play a central role in cellular homeostasis [47]. To our knowledge, this is the first study demonstrating the down-regulation of *hsp70* gene in skin under metals stress and within short acute exposure. Nevertheless, Xin and his collaborator [72] described a significant down-regulation in the mRNA expression level of *hsp70* in gill and liver of *Plecoglossus altivelis* exposed to slain variation stress condition after long-time exposure and suggested that the change of *hsp70* may participate in osmoregulation, metabolism, and nutrient storage.

The analysis of the expression of *cyp1a1* gene in this study showed an up-regulation in the liver of specimens reared in black and mixed sediments. This up-regulation reached significant level in specimens reared with mixed sediments. The family of *CYP1a* are haem-containing enzymes, which catalyze various Phase I metabolism reactions, such as C-, N- and S- oxidation and dealkylation. The transcriptional activation of the *cyp1a1* gene is mediated by the binding of environmental pollutants and inhalation chemicals [66]. Anwar et al. [4] demonstrated that *cyp1a1* activity, regarding to As, does not always parallel its effect on the expression on *cyp1a1* mRNA. In the same study the author suggested that the activity of *cyp1a1* was accompanied either by a decrease or no change in mRNA levels. In contrast, Pb^{2+} alone was able to increase *cyp1a1* mRNA levels without affecting its protein levels, and Cd^{2+} did not affect *cyp1a1* mRNA stability [4]. Thus, we cannot confirm, in this study, if this induction (up-regulation) of *cyp1a1* gene is activated by one specific heavy metal or it is the consequence of exposure to all heavy metals existing in the sediments. In *Platichthys flesus*, Cd has been shown to inhibit *cyp1a* activity but this was thought to be post-translational [4].

5. Conclusions

The Portman Bay sediments are highly concentrated in heavy metals. Exposure of gilthead seabream to black, yellow and mixed sediments demonstrate that accumulation of heavy metals in fish depend on both metal and organ studied. Only Cd accumulates in liver while no metals accumulation was revealed in both studied edible organs (skin and muscle). The expression of *mta*, *hsp70* and *hsp90* genes was modified in skin but not in liver. In contrast, *cyp1a1* gene was induced in liver cells and not in skin. The induction of *mta*, *hsp70* and *hsp 90* genes in skin after 15 days of exposure of gilthead seabream allow this organ to be probably the suitable organ for the encouraged study of biomarkers genes expression. Interestingly, skin could be sampled without killing the fish specimen; this fact could be very useful for fish farms to control the wellbeing of fish.

Sediments are an important habitat component in aquatic ecosystems, as they provide a substrate or support medium for aquatic organisms. This study could be considered very interesting for a potential biological restoration plan in Portman Bay to reactivate the biological activity and establish the normal situation in sediments using gilthead seabream in order to restore the biota in the place. Further experiments could be made using other elements of the trophic chain such as phytoplankton, zooplankton, in order to elucidate potential bioaccumulation of heavy metals and whether this could contribute to significant effects on the food chains by bioaccumulation.

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References

[1] A.S. Abdel-Baki, M.A. Dkhil, S. Al-Quraishy, Bioaccumulation of some heavy metals in tilapia fish relevant to their concentration in water and sediment of Wadi Hanifah, Saudi Arabia, Afr. J. Biotechnol. 10 (2011) 2541–2547.

[2] Z. Abedi, F. Hasantabar, A. Mohammad, K. Khalesi, S. Babaei, Effect of sublethal concentrations of cadmium, lead and chromium on some enzymatic activities of common carp; *Cyprinus carpio*, World J. Zool. 8 (2013) 98–105.

[3] A. Annabi, K. Said, I. Messaoudi, Cadmium: bioaccumulation, histopathology and detoxifying mechanisms in fish, Am. J. Res. Com. 1 (2013) 60–79.

[4] M.A. Anwar, H.R. Elbekai, A.O. El-Kadi, Regulation of CYP1A1 by heavy metals and consequences for drug metabolism, Expert Opin. Drug Metab. Toxicol. 5 (2009) 501–521.

[5] J.M. Arellano, V. Storch, C. Sarasquete, Histological changes and copper accumulation in liver and gills of the Senegalese sole: *Solea senegalensis*, Ecotoxicol. Environ. Saf. 44 (1999) 62–72.

[6] B. Baldisserotto, C. Kamunde, A. Matsuo, C.M. Wood, A protective effect of dietary calcium against acute waterborne cadmium uptake in rainbow trout, Aquat. Toxicol. 67 (2004) 57–73.

[7] L. Bargelloni, R. Scudiero, E. Parisi, V. Carginale, C. Capasso, T. Patarnello, Metallothioneins in antarctic fish: evidence for independent duplication and gene conversion, Mol. Biol. Evol. 16 (1999) 885–897.

[8] S. Benhamed, F.A. Guardiola, M. Mars, M.A. Esteban, Pathogen bacteria adhesion to skin mucus of fishes, Vet. Microbiol. 171 (2014) 1–12.

[9] H.D. Belitz, W. Grosch, Food Chem., 2nd ed., Springer, Berlin, 1999.

[10] L. Bervoets, D. Knapen, M. De Jonge, K. Van Campenhout, R. Blust, Differential hepatic metal and metallothionein levels in three feral fish species along a metal pollution gradient, PLoS One 8 (2013) 1–11.

[11] A. Bhattacharya, S. Bhattacharya, Induction of oxidative stress by arsenic in *Clarias batrachus*: involvement of peroxisomes, Ecotoxicol. Environ. Saf. 66 (2007) 178–187.

[12] J. Bloesch, 2009. Sediments of aquatic ecosystems, encyclopedia of inland water reference module in earth systems and environmental sciences. 1, 479–490.

[13] J.C. Brodeur, C. Daniel, A.C. Ricard, A. Hontela, In vitro response to ACTH of the interrenal tissue of rainbow trout (*Oncorhynchus mykiss*) exposed to cadmium, Aquat. Toxicol. 42 (1998) 103–113.

[14] L. Coetzee, H.H. du Preez, J.H.J. Van Vuren, Metal concentrations in *Clarias gariepinus* and *Labeo umbratus* from the Olifants and Klein Olifants River, Mpumalanga, South Africa : zinc, copper, manganese, lead, chromium, nickel, aluminium and iron, Water SA 28 (4) (2002) 433–448.

[15] K.M. Chan, Metallothionein: potential biomarker for monitoring heavy metal pollution in fish around Hong Kong, Mar. Pollut. Bull. 31 (1995) 411–415.

[16] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, Biotechniques 15 (1993) 532–537.

[17] R. Cornelis, 2005. Handbook of Elemental Speciation 481 II–Species in the Environment, Food. In: Handbook of Elemental Speciation II: Species in the Environment, Food, Medicine and Occupational Health, R. Cornelis, J. Caruso, H. Crews, K.G. Heumann (Eds.), England, pp. 0–470.

[18] P. Coyle, J.C. Philcox, L.C. Carey, A.M. Rofe, Metallothionein: the multipurpose protein, Cell. Mol. Life Sci. 59 (2002) 627–647.

[19] A.J. Dangre, S.B. Manning, Effects of cadmium on hypoxia-induced expression of hemoglobin and erythropoietin in larval sheepshead minnow *Cyprinodon variegates*, Aquat. Toxicol. 99 (2010) 168–175.

[20] S. Datta, D.R. Saha, D. Ghosh, T. Majumdar, S. Bhattacharya, S. Mazumder, Sub-lethal concentration of arsenic interferes with the proliferation of hepatocytes and induces in vivo apoptosis in *Clarias batrachus* L, Comp. Biochem. Physiol. C 145 (2007) 339–349.

[21] M.A. Esteban, J. Meseguer, The phagocytic defence mechanism in sea bass (*Dicentrarchus labrax* L.) an ultrastructural study, Anat. Rec. 240 (1994) 589–597.

[22] M.A. Esteban, An overview of the immunological defenses in fish skin, ISRN Immunol. 2012 (2012) 1–29.

[23] A. Giardina, S.E. Larson, B. Wisner, J. Wheeler, M. Chao, Long-term and acute effects of zinc contamination of a stream on fish mortality and physiology, Environ. Toxicol. Chem. 28 (2009) 287–295.

[24] A.W. Glynn, Cadmium and zinc kinetics in fish: studies on water-borne 109Cd and 65Zn turnover and intracellular distribution in minnows *Phoxinus phoxinus*, Pharmacol. Toxicol. 68 (1991) 485–491.

[25] F.A. Guardiola, M.P. Gónzalez-Párraga, A. Cuesta, J. Meseguer, S. Martínez, M.J. Martínez-Sánchez, C. Pérez-Sirvent, M.A. Esteban, Immunotoxicological effects of inorganic arsenic on gilthead seabream (*Sparus aurata* L.), Aquatic Toxicol. 134–135 (2013) 112–119.

[26] K. Güven, C. Özbay, E. Ünlü, A. Satar, Acute lethal toxicity and accumulation of copper in *Gammarus pulex* (L.) (Amphipoda), Turk. J. Biol. 23 (1999) 513–521.

[27] R.D. Handy, The assessment of episodic metal pollution: i. Uses and limitations of tissue contaminant analysis in rainbow trout (*Oncorhynchus mykiss*) after short waterborne exposure to cadmium or copper, Arch. Environ. Contam. Toxicol. 22 (1992) 74–81.

[28] J.B. Harford, R.D. Klausner, Coordinate post-transcriptional regulation of ferritin and transferrin receptor expression: the role of regulated RNA-protein interaction, Enzyme 44 (1990) 28–41.

[29] M.L. Haude, D. Jocelyn, H. Alice, J.V. Glen, G.C.C. Peter, Hormonal, morphological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures, J. Toxicol. Environ. Health A 66 (2003) 657–676.

[30] M.S. Heydarnejad, M. Khosravian-Hemamai, A. Nematollahi, Effects of cadmium at sub-lethal concentration on growth and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*), Ir. Vet. J. 66 (2013) 2–7.

[31] A. Hontella, C. Daniel, A.C. Ricard, Effects of acute and subacute exposures to cadmium on interrenal and thyroid function in rainbow trout *Oncorhynchus mykiss*, Aquat. Toxicol. 35 (1996) 171–182.

[32] F. Jabeen, A.S. Chaudhry, Monitoring trace metals in different tissues of *Cyprinus carpio* from Indus River in Pakistan, Environ. Monit. Assess. 170 (2010) 645–656.

[33] M. Javed, N. Usmani, I. Ahmad, M. Ahmad, Studies on the oxidative stress and gill histopathology in *Channa punctatus* of the canal receiving heavy metal loaded effluent of Kasimpur Thermal Power Plant, Environ. Monit. Assess. 187 (2015) 1–11.

[34] M. Javed, I. Ahmad, N. Usmani, M. Ahmad, Bioaccumulation, oxidative stress and genotoxicity in fish (*Channa punctatus*) exposed to a thermal power plant effluent, Ecotoxicol. Environ. Saf. 127 (2016) 163–169 http://dx.doi.org/10.1016/j.ecoenv.2016.01.007.

[35] M. Javed, N. Usmani, Accumulation of heavy metals in fishes: a human health concern, Int. J. Environ. Sci. 2 (2011) 659–670.

[36] M. Javed, N. Usmani, Assessment of heavy metals (Cu, Ni, Fe, Co, Mn, Cr, Zn) in rivulet water, their accumulations and alterations in hematology of fish *Channa punctatus*, Afr. J. Biotechnol. 13 (3) (2014) 492–501.

[37] M. Javed, N. Usmani, Stress response of biomolecules (carbohydrate, protein and lipid profiles) in fish *Channa punctatus* inhabiting river polluted by Thermal Power Plant effluent, Saudi J. Biol. Sci. 22 (2015) 237–242.

[38] H. Karadede, S.A. Oymak, E. Unlu, Heavy metals in mullet, *Liza abu*, and catfish Silurus triostegus, from the Ataturk Dam Lake (Euphrates) Turkey, Environ. Int. 30 (2004) 183–188.

[39] R. Klaus, H. Martin, B. Johannes, The heat shock response: life on the verge of death, Mol. Cell 40 (2010) 253–266.

[40] J. Kojadinovic, M. Potier, M. Le Corre, R.P. Cosson, P. Bustamante, Bioaccumulation of trace elements in pelagic fish from the Western Indian Ocean, Environ. Pollut. 146 (2007) 548–566.

[41] M.H. Kraal, M.H. Kraak, C.J. de Groot, C. Davids, Uptake and tissue distribution of dietary and aqueous cadmium by carp (*Cyprinus carpio*), Ecotoxicol. Environ. Saf. 31 (1995) 179–183.

[42] A. Lacroix, A. Hontela, A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch *Perca flavescens*, Aquat. Toxicol. 67 (2004) 13–21.

[43] S. Lal Shah, Hematological changes in *Tinca tinca* after exposure to lethal and sublethal doses of mercury cadmium and lead, Iran. J. Fish. Sci. 9 (2013) 434–443.

[44] C.B.R. Martinez, M.Y. Nagae, C.T.B.V. Zaia, D.A.M. Zaia, Acute morphological and physiological effects of lead in the neotropical fish *Prochilodus lineatus*, Braz. J. Biol. 64 (2004) 797–807.

[45] M.J. Martínez-Sánchez, M.C. Navarro, C. Pérez-Sirvent, J. Marimón, J. Vidal, M.L. García-Lorenzo, J. Bech, Assessment of the mobility of metals in a mining-impacted coastal area (Spain, Western Mediterranean), J. Geochem. Explor. 96 (2008) 171–182.

[46] M.J. Martínez-Sánchez, C. Pérez-Sirvent, M.L. García, S. Martínez, J.M. Veiga, E. González, V. Pérez, L.B. Martínez, C. Hernández, 2013. Las Arenas de la Bahía de Portman: Residuo Mena de Hierro. Macla no. 17. Revista de la Sociedad Española de Mineralogía.

[47] Y. Michiaki, Y. Takeshi, O. Nobuhiko, Stress protein HSP70 in fish, Aqua-BioSci Monogr. 3 (2010) 111–141.

[48] J.A. McCarter, A.T. Matheson, M. Roch, R.W. Olafson, J.T. Buckley, Chronic exposure of coho salmon to sublethal concentrations of copper II: distribution of copper between high and low-molecular weight proteins in the liver cytosol and the possible role of metallothionein in detoxification, Comp. Biochem. Physiol. 74C (1982) 133–137.

[49] J.C. McGeer, C. Szebedinszky, D.G. McDonald, C.M. Wood, Effects of chronic sub lethal exposure to waterborne Cu, Cd, or Zn in rainbow trout: 1: iono-regulatory disturbance and metabolic costs, Aquat. Toxicol. 50 (2000) 233–245.

[50] A.L. Nick, D.W. Tim, K.C. James, Functional analysis of a metal response element in the regulatory region of flounder cytochrome P450 1A and implications for environmental monitoring of pollutants, Toxicol. Sci. 92 (2006) 387–393.

[51] P.E. Olsson, Disorders associated with heavy metal pollution, in: J.E. Leatherland, P.T.K. Woo (Eds.), Fish Diseases and Disorders Volume 2 (non-infectious Disorders), CABI International, U.K, 1998, pp. 105–131.

[52] R.M. Pedlar, M.D. Ptashynski, K.G. Wautier, R.E. Evans, C.L. Baron, J.F. Klaverkamp, The accumulation, distribution, and toxicological effects of dietary arsenic exposure in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*), Comp. Biochem. Physiol. C. 131 (2002) 73–91.

[53] C. Pérez-Sirvent, M. Luz Garcia-Lorenzo, M.J. Martinez-Sanchez, J. Molina-Ruiz, J. Marimon, M. Cortes Navarro, Use of marble cutting sludges for remediating soils and sediments contaminated by heavy metals, Environ. Prog. Sust. Energ. 30 (2010) 533–539.

[54] K. Puneet, S. Anu, Cadmium toxicity in fish: an overview, GERF. Bull. Biosci. 1 (2010) 41–47.
[55] N. Rangsayatorn, M. Kruatrachue, P. Pokethitiyook, E.S. Upatham, G.R. Lanza, S. Singhakaew, Ultrastructural changes in various organs of the fish *Puntius gonionotus* fed cadmium-enriched cyanobacteria, Environ. Toxicol. 19 (2004) 585–593.
[56] A.C. Ricard, C. Daniel, P. Anderson, A. Hontela, Effects of subchronic exposure to cadmium chloride on endocrine and metabolic functions in rainbow trout *Oncorhynchus mykiss*, Arch. Environ. Contam. Toxicol. 34 (1998) 377–381.
[57] L. Robaina, F.J. Moyano, M.S. Izquierdo, J. Socorro, J.M. Vergara, D. Montero, Corn gluten and meat and bone meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications, Aquaculture 157 (1997) 347–359.
[58] M. Romeo, Y. Siau, Z. Sidoumou, M. Gnassia-Barelli, Heavy metal distribution in different fish species from the Mauritania coast, Sci. Total Environ. 232 (1999) 169–175.
[59] B. Sana, M. Imed, D. Tmim, S. Khaled, K. Abdelhamid, Cadmium bioaccumulation in three benthic fish species, *Salaria basilisca*, *Zosterisessor ophiocephalus* and *Solea vulgaris* collected from the Gulf of Gabes in Tunisia, J. Environ. Sci. 21 (2009) 980–984.
[60] T. Sakurai, T. Ohta, N. Tomita, C. Kojima, Y. Hariya, A. Mizukami, K. Fujiwara, Evaluation of immunotoxic and immunodisruptive effects of inorganic arsenite on human monocytes/macrophages, Int. Immunopharmacol. 6 (2006) 304–315.
[61] S.A. Shaffi, Y.R. Manohar, M.J. Nandan, Influence of protective agents on metal induced respiratory distress in *Labeo rohita* (Ham), Bull. Environ. Contam. Toxicol. 66 (2001) 611–616.
[62] P. Sivaperumal, T. Sankar, P. Viswanathannair, Heavy metal concentrations in fish, shellfish and fish products from internal markets of India vis-à-vis international standards, Food Chem. 102 (2007) 612–620.
[63] D. Soma, R.S. Dhira, G. Debabrata, M. Tanmay, B. Shelley, M. Shibnath, Sub-lethal concentration of arsenic interferes with the proliferation of hepatocytes and induces in vivo apoptosis in *Clarias batrachus* L, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 145 (2007) 339–349.
[64] K. Uysal, Y. Emre, E. Köse, The determination of heavy metal accumulation ratios in muscle, skin and gills of some migratory fish species by inductively coupled plasma-optical emission spectrometry (ICP-OES) in Beymelek Lagoon (Antalya/Turkey), Microchem. J. 90 (2008) 67–70.
[65] J.C. Van Dyk, G.M. Pieterse, J.H. van Vuren, Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc, Ecotoxicol. Environ. Saf. 66 (2007) 432–440.
[66] P.A. Vasilis, M.T. Aristidis, A.S. Demetrios, Cytochrome P450CYP1A1: wider roles in cancer progression and prevention, BMC Cancer 9 (2009) 187.
[67] W.X. Wang, Interactions of trace metals and different marine food chains, Mar. Ecol. Prog. Ser. 243 (2002) 295–309.
[68] S. Vineeta, D. Monika, P. Jai, K.V. Sastry, Bioaccumulation of Zn, Cu and Cd in *Channa punctatus*, J. Environ. Biol. 2 (2007) 395–397.
[69] M. Witeska, B. Jezierska, J. Wolnieki, Respiratory and hematological response of tench *Tinca tinca* (L:) to a short-term cadmium exposure, Aquacul. Int. 14 (2006) 141–152.
[70] C. Wu, Heat shock transcription factors: structure and regulation, Annu. Rev. Cell Dev. Biol. 11 (1995) 441–469.
[71] M.M. Wu, H.Y. Chiou, I.C. Ho, C.J. Chen, T.C. Lee, Gene expression of inflammatory molecules in circulating lymphocytes from arsenic-exposed human subjects, Environ. Health Perspect. 111 (2003) 1429–1438.
[72] J.L. Xin, C. Jiong, A.H. Zuo, H.S. Yu, W. Fang, Proteomic analysis on the alteration of protein expression in gills of ayu (*Plecoglossus altivelis*) associated with salinity change, Comp. Biochem. Physiol. D 5 (2010) 185–189.
[73] A.B. Yilmaz, Levels of heavy metals (Fe, Cu, Ni, Cr, Pb, and Zn) in tissue of mugil cephalus and trachurus mediterraneus from iskenderun bay, Turk. Environ. Res. 92 (2003) 277–281.
[74] R.V. Zikic, A.S. Stajn, S.Z. Pavlovic, B.I. Ognjanovic, Z.S. Saicic, Activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of goldfish (*Carassius auratusgibelio* Bloch) exposed to cadmium, Physiol. Res. 50 (2001) 105–111.