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Inorganic arsenic causes apoptosis cell death and immunotoxicity on European sea bass (*Dicentrarchus labrax*)

Héctor Cordero[a], Patricia Morcillo[b], Salvadora Martínez[c], José Meseguer[a], Carmen Pérez-Sirvent[c], Elena Chaves-Pozo[d], María José Martínez-Sanchez[c], Alberto Cuesta[a], M. Ángeles Esteban[a,*]

[a] Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain
[b] Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, 10461, NY, USA
[c] Department of Agricultural Chemistry, Geology and Pedology, Faculty of Chemistry, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain
[d] Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera de la Azohía s/n., 30860 Puerto de Mazarrón, Murcia, Spain

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ABSTRACT

Inorganic arsenic (As) is one of the most toxic pollutants in the water. We have studied their effects on the marine teleost European sea bass (*Dicentrarchus labrax*) at 2 and 10 days of 5 μM of As_2O_3 (sub-lethal doses) waterborne exposure. Arsenic accumulates in liver and gill tissues. The expression profile of five genes (*bax*, *blc2*, *casp3*, *casp8* and *casp9*) involved in apoptosis cell death confirmed apoptotic effects in liver, slight changes in gill and no effects in skin according with the histopathology findings. Total IgM level and peroxidase activities were increased at 2 and 10 days, respectively. The bactericidal activity was decreased at 2 days after As exposure. A general decrease of cellular immune activities with significant differences in the case of respiratory burst activity was observed after 2 and 10 days of exposure. This work describes for the first time the effects of As exposure on European sea bass.

1. Introduction

Contamination of aquatic habitats with metals and metalloids from various industrial and mineral mining sources is a really serious environmental problem (Duker et al., 2005; Morcillo et al., 2015b). As a metalloid, Arsenic (As) can be found in both inorganic and organic forms. In addition, this metalloid has different valence states, being trivalent (As^{+3}) and pentavalent (As^{+5}) states the most impacting in the environment (Hughes, 2002) and As^{+3} the most toxicologically potent (Hughes et al., 2011). The bioaccumulation of As in aquatic organisms and especially in fish is dangerous not only for their own life, but also for humans since fish are known to be the greatest inputs of toxic trace elements for humans (EFSA, 2005). The relevance of European sea bass (*Dicentrarchus labrax*) is due to the high commercial value mainly in Europe (FAO, 2014).

The toxicological effects of As exposure are relatively well studied in human. As has been reported to cause keratosis (Mazumder et al., 1998), carcinoma (Ishinishi et al., 1977; Tsuji et al., 2014), DNA damage with production of reactive oxygen species (ROS) (Barchowsky et al., 1999; Kitchin et al., 1999), oxidative stress and apoptosis (De La Fuente et al., 2002). By contrast, most of the studies in fish are focused on liver alterations, including effects on cell proliferation and oxidative stress (Bhattacharya and Bhattacharya, 2007; Carlson and Van Beneden, 2014; Datta et al., 2007; Kotsanis and Iliopoulou-Georgudaki, 1999) as well as antioxidant imbalance inducing heat shock proteins (Bears et al., 2006; Lam et al., 2006; Del Razo et al., 2001). However, the study of the mucosal tissues such as gills and skin, which are constantly exposed to As waterborne, are less studied.

On the other hand, its effects on immune function are also less understood, especially in aquatic species (Lage et al., 2006). Aquatic contamination due to metalloids such as As causes strong effects on fish health, increasing the susceptibility to infection and in many cases causing death ultimately. Few species such as *Danio rerio* (Hermann and Kim, 2005; Nayak et al., 2007), *Clarias batrachus* (Ghosh et al., 2006), *Labeo rohita* (Banerjee et al., 2015) or *Sparus aurata* (Guardiola et al., 2013, 2015) have been studied *in vivo*. In addition, As-exposure *in vitro* produces a selective head-kidney macrophages death (Datta et al., 2009a, 2009b), down-regulates the synthesis of macrophage-derived

* Corresponding author.
E-mail address: aesteban@um.es (M. Ángeles Esteban).

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cytokines such as *tumour necrosis factor* (*tnfa*) and *interferon gamma* (*ifng*) (Lage et al., 2006) and decreases the phagocytic activity of macrophages (Ghosh et al., 2006). In the case of *D. labrax*, the immunotoxicological effects of As_2O_3 have been also tested *in vitro* (Morcillo et al., 2015a) but not their effects *in vivo*. Since very little is known about the specific effects of As_2O_3 on European sea bass (*D. labrax*) and their repercussions at higher trophic levels by consumption, the aim of this work was to evaluate the accumulation, tissue histopathological alterations, including apoptosis, necrosis, and the immune response by waterborne exposure to As.

2. Materials and methods

2.1. Fish maintenance

Twenty-four specimens (97.3 ± 13.2 g body weight and 18.6 ± 1.8 cm body-length) of European sea bass (*D. labrax*), obtained from *Instituto Español de Oceanografía* (Murcia, Spain), were kept for 15 days before starting the experimental trial (acclimation) in seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water was maintained at 20 ± 2 °C with a closed flow rate of 900 l h^{-1}, 28‰ salinity, pH 7.7, and nitrates 25 ppm. The photoperiod was of 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day^{-1}. They were starved for 24 h prior to sampling and sacrificed by an overdose of MS222 (Sandoz, 100 mg ml^{-1} water) (Esteban and Meseguer, 1994). All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Arsenic exposure

Arsenic trioxide (As_2O_3; Fluka Analytical) was prepared as describe elsewhere (Ghosh et al., 2006) with some modifications. Thus, As_2O_3 was dissolved in acidic water containing 1 N HCl with occasional stirring. Once it was completely dissolved, the pH was adjusted to 7.2 by drop wise addition of 1 N NaOH and filtered by passing through 0.2 μm filters. Twenty-four specimens were randomly divided into four tanks (six in each tank), two tanks remained unexposed (control group) and two tanks were exposed to 5 μM of As_2O_3.

2.3. Fish sampling

Four fish per group and time point (two fish from each tank; $n = 4$) were sampled after 2 and 10 days of As-exposure. First, specimens were dissected and weighted. Fragments of gill, skin and liver were stored at −80 °C for total As determination, processed for histology or for RNA purification. Blood samples were collected from the caudal vein with an insulin syringe. After clotting at 4 °C for 4 h, serum was collected after centrifugation (9,500*g*, 5 min) and stored at −80 °C until use to make sure the best levels of humoral activities as described elsewhere (Cordero et al., 2016a). Head-kidney (HK) fragments were transferred to 8 ml sRPMI-1640 culture medium [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride to adjust the medium's osmolarity to European sea bass plasma osmolarity of 353.33 mOs], 3% (v/v) foetal calf serum (FCS, Life Technologies), 10 IU ml^{-1} heparin (Sigma-Aldrich), 100 IU ml^{-1} penicillin (Life Technologies) and 100 μg ml^{-1} streptomycin (Life Technologies) (Esteban et al., 1998). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm), washed twice in sRPMI (400*g*, 10 min), counted (Z2 Coulter Particle Counter) and adjusted to 10^7 cells ml^{-1} in sRPMI.

2.4. Analysis of total arsenic in tissues

Frozen gill, skin and liver samples 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), since it has been demonstrated to be an efficient method for determining total As (Shah et al., 2009). Finally, 50 μl of the solution was used to determine the arsenic 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 As per kg dry-weight tissue.

2.5. Light microscopy

Liver, skin and gill samples were fixed with 4% neutral buffered formaldehyde (Panreac) for 24 h. After serial dehydration steps in alcohol, samples were embedded in paraffin (Thermo Scientific), sectioned at 5 μm, mounted and stained with haematoxylin-eosin (H-E). Slides were analyzed by a light microscope (Leica 6000B) and images were acquired with Leica DFC280 digital camera.

2.6. Gene expression analysis

Around 50 mg of liver, skin and gill samples ($n = 4$) were extracted with TRIzol® reagent (Life Technologies) following the manufacturer's instructions, quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8–2.0. In addition, 1 μl of each RNA sample was run on 2% agarose gel to check the integrity. Next, RNA was treated with DNase I (Promega) to remove genomic DNA. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer (Life Technologies). Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) with SYBR Green PCR Core Reagents (Applied Biosystems) as described elsewhere (Cordero et al., 2015) and using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Efficiency of PCR was calculated according to Cordero et al. (2016b). Normalization factors were calculated as the geometric mean of relative quantities of reference genes elongation factor 1 alpha (*ef1a*) and actin beta (*actb*) according to the Pfaffl method (Pfaffl et al., 2004). Negative controls had no amplification product and control templates showed no primer-dimer formations. The *bcl2* sequence and its accession number were obtained from European sea bass (Nuñez-Ortiz et al., 2014). More information about primers is detailed in Table 1.

2.7. Immune parameters

2.7.1. Serum IgM assay

Total serum IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) (Cuesta et al., 2004). Thus, 20 μl per well of 1/100 fold diluted serum were placed in flat-bottomed 96-well plates in triplicate and the protein coating was performed by overnight incubation at 4 °C with 200 μl carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM Na_2CO_3, pH 9.6). After three rinses with PBT [containing PBS (Sigma-Aldrich) and 0.05% Tween 20 (Fluka)] the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBT, followed by three rinses with PBT. The plates were then incubated for 1 h with 100 μl per well of mouse anti-European sea bass IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with secondary antibody anti-mouse IgG-HRP (1/1000 in blocking buffer; Sigma-Aldrich). After exhaustive rinsing with PBT the plates were developed using 100 μl 0.42 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma-Aldrich) solution,

Table 1
Primers used in the qPCR study.

Gene[a]	AN[b]	E(%)[c]	R^{2d}	Primers
actb	AJ537421	97.1	0.997	F: TCCCTGGAGAAGAGCTACGA
				R: AGGAAGGAAGGCTGGAAAAG
ef1a	AJ866727	97.3	0.997	F: CGTTGGCTTCAACATCAAGA
				R: GAAGTTGTCTGCTCCCTTGG
bax	FM011848	104.0	0.998	F: TGTCGACTCGTCATCAAAGC
				R: CACATGTTCCCGGAGGTAGT
bcl2	KX065053	99.4	0.990	F: GACTGTACCAGCCGGACTTC
				R: GTCCCGGAACAGTTCGTCTA
casp3	DQ345773	87.5	0.980	F: AATTCACCAGGCTTCAATGC
				R: CTACGGCAGAGACGACATCA
casp8	FJ225665	104.7	0.992	F: ACACGTGTGAACAGGGAGGT
				R: TTGAGGACGAGCTTCTTGGT
casp9	DQ345775	102.2	0.993	F: AACGAGTGGGGTTGTTTCAG
				R: ATGGGTCCAAGTCTCTCACG

[a] Gene symbol according to zebrafish nomenclature (http://zfin.org/).
[b] Accession number according to NCBI database.
[c] Efficiency of the qPCR reaction in percentage (%).
[d] Correlation index in the standard curve of each gene.

Table 2
Concentration of inorganic As (μg kg dry-weight^{-1}) determined in gill, skin and liver tissues from European sea bass (*D. labrax*) specimens unexposed or exposed to 5 μM of As_2O_3. ql: quantification limit (0.1 μg kg dry-weight^{-1}). Results are expressed as mean ± SEM (n = 4).

Target tissue	Group	Exposure time	
		2 days	10 days
Liver	Unexposed	< ql	< ql
	Exposed	< ql	582 ± 59
Skin	Unexposed	< ql	< ql
	Exposed	< ql	< ql
Gill	Unexposed	< ql	< ql
	Exposed	1050 ± 121	1214 ± 119

prepared daily in distilled water containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 μl 2 M H_2SO_4 and the plates were read at 450 nm in a plate reader (BMG, Fluostar Omega). Negative control consisted of samples without serum or without primary antibody by triplicate, whose OD values were subtracted for each sample value.

2.7.2. Antiprotease activity

Total antiprotease activity was determined as indicated by the capacity of serum to inhibit trypsin activity (Hanif et al., 2004). Briefly, 20 μl of serum was incubated with 20 μl of standard trypsin solution (5 mg μl^{-1}; Sigma–Aldrich) for 10 min at 22 °C in Eppendorf tubes. Then, 200 ml of 0.1 M PBS (pH 7.0) and 250 μl of 2% (w/v) azocasein (Sigma–Aldrich) in PBS were added, and incubated for 1 h at 22 °C. The reaction was stopped by the addition of 500 μl of 10% (v/v) trichloroacetic acid (TCA, Sigma–Aldrich), incubated for 30 min at 22 °C, and then centrifuged at 6000*g* for 5 min. The supernatants (100 μl) were transferred to a 96-well microtiter plate (Nunc) containing 100 μl well^{-1} of 0.5 N sodium hydroxide (NaOH). The OD was read at 450 nm using a plate reader. For a positive (100%) control, buffer replaced the serum, and for a negative control, buffer replaced both serum and trypsin. The inhibitory ability of antiprotease was expressed in terms of percentage trypsin inhibition according to the formula:

$$\%\text{Trypsin inhibition} = \text{Trypsin OD} - \text{Sample OD}/\text{Trypsin OD} \times 100$$

2.7.3. Natural heamolytic complement activity

The alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets according to Ortuño et al. (1998). Briefly, 100 μl of SRBC suspension (6%) in phenol red-free

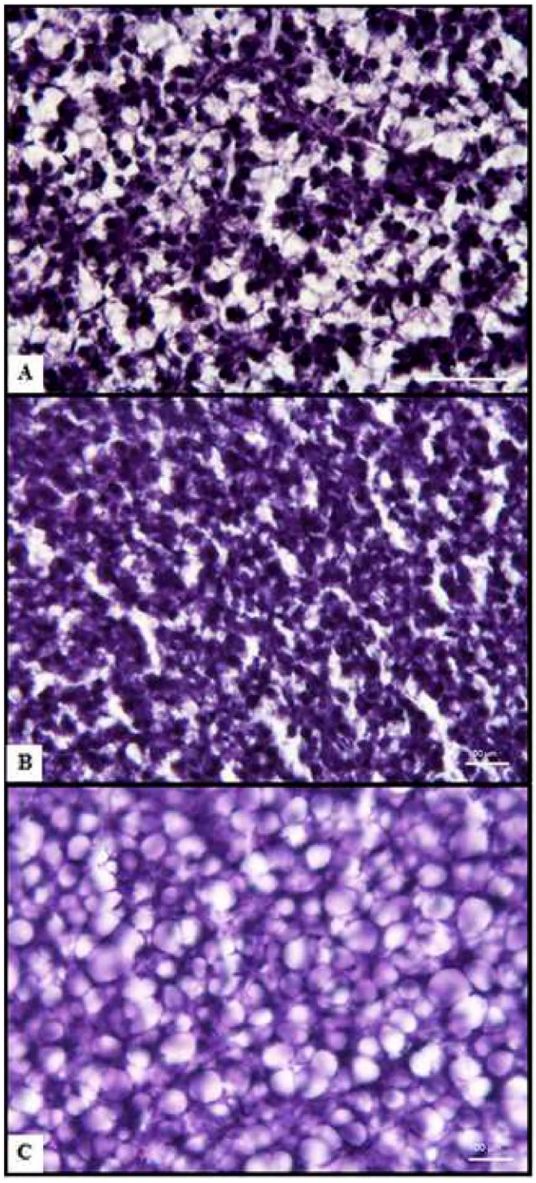

Fig. 1. Representative histological images from liver stained with haematoxylin-eosin from European sea bass specimens unexposed (A) or exposed to 5 μM of As_2O_3 at 2 (B) and 10 (C) days. Arrows indicate apoptotic and/or necrotic symptoms.

Hank's buffer (HBSS) (Life Technologies) containing Mg^{+2} and EGTA were mixed with 100 μl of serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation (90 min, 22 °C), the samples were centrifuged (400*g*, 5 min, 4 °C) to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a plate reader. The values of maximum (100%) and minimum haemolysis were obtained by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively.

The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting $Y/(1 - Y)^{-1}$ against the volume of serum added (ml) on a log-log scale graph. The volume of serum producing 50% haemolysis (ACH_{50}) and the number of ACH_{50} units ml^{-1} obtained for each experimental group were determined.

2.7.4. Bactericidal activity

Bactericidal assay was conducted with a non-pathogenic bacterium (*Escherichia coli*), one fish opportunist pathogenic bacterium (*Vibrio harveyi)*. Bacteria cells were grown in agar plates with Luria Broth (LB,

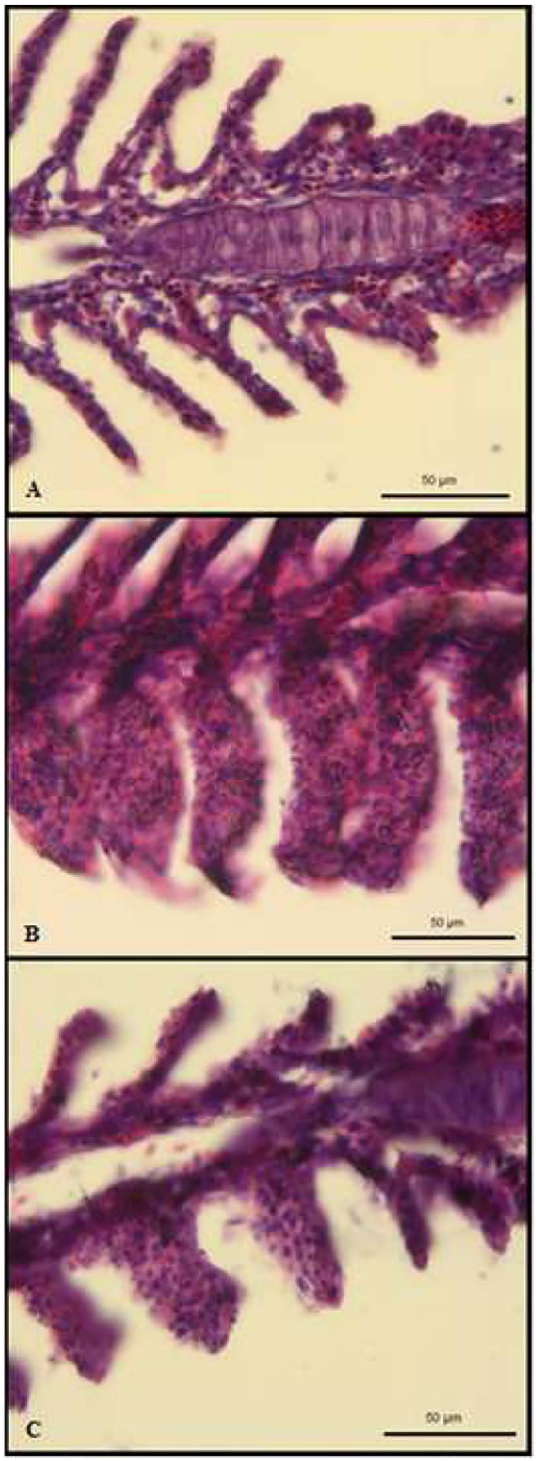

Fig. 2. Representative histological images from gills stained with haematoxylin-eosin from European sea bass specimens unexposed (A) or exposed to 5 µM of As_2O_3 at 2 (B) and 10 (C) days. Hypertrophy and thickening or fusion of lamellae are seen.

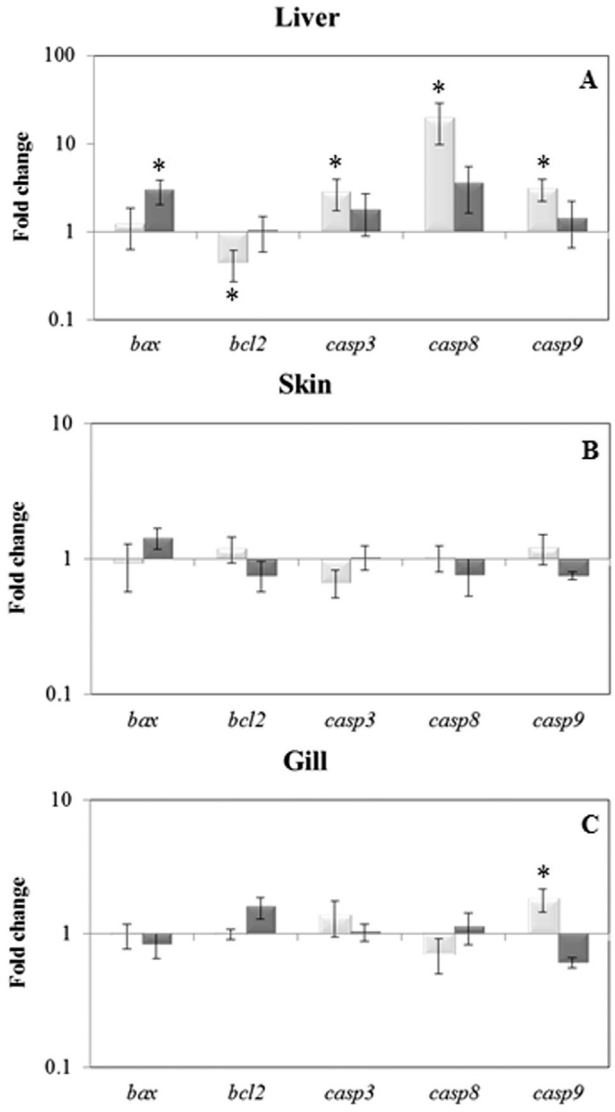

Fig. 3. Gene expression profile of apoptotic genes (*bax*, *bcl2*, *casp3*, *casp8* and *casp9*) in the liver, skin and gills of European sea bass specimens exposed to 5 µM of As_2O_3 at 2 days (white bars) and 10 days (black bars). Data are expressed as gene expression fold change with respect to the time-matched unexposed groups. Bars are represented as mean ± SEM (n = 4). Asterisks denote significant differences between exposed and unexposed groups according to the Student *t*-test when $p < 0.05$.

Sigma-Aldrich) for *E. coli*, and Tryptic Soy Broth (TSB, Sigma-Aldrich) supplemented with 1.5% NaCl for both *V. harveyi* at 22 °C. Then, fresh single colonies of 1–2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 16 h at 22 °C on an orbital incubator at 250 rpm. Serum antimicrobial activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort (Sunyer and Tort, 1995) with some modifications. Aliquots of 100 µl of each one of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of sea bass serum. The OD of the samples was measured at 620 nm at 30 min intervals during 24 h at 22 °C. Serum samples without bacteria were used as blanks (100% bactericidal activity), and bacterial samples without serum were used as positive controls (0% bactericidal activity). Samples and controls were conducted by triplicated.

2.7.5. Respiratory burst activity

The respiratory burst activity of sea bass HKL was studied by a chemiluminescence method (Bayne and Levy, 1991). For this assay, 100 µl of HK leucocyte suspension (10^6 HK leucocytes) were placed in triplicate in wells of a 96-well flat-bottomed plate. Then, 100 µl of HBSS (Hank's balanced salt solution, Gibco) containing 1 µg ml^{-1} phorbol myristate acetate (PMA, Sigma-Aldrich) and 10^{-4} M luminol, (Sigma-Aldrich) were added to each well. The plates were shaken and immediately read in a plate reader for 1 h at 2 min intervals. The kinetic of the reactions was analyzed and the maximum slope of each curve calculated. Backgrounds of luminescence were calculated using reactant solutions containing luminol but not PMA.

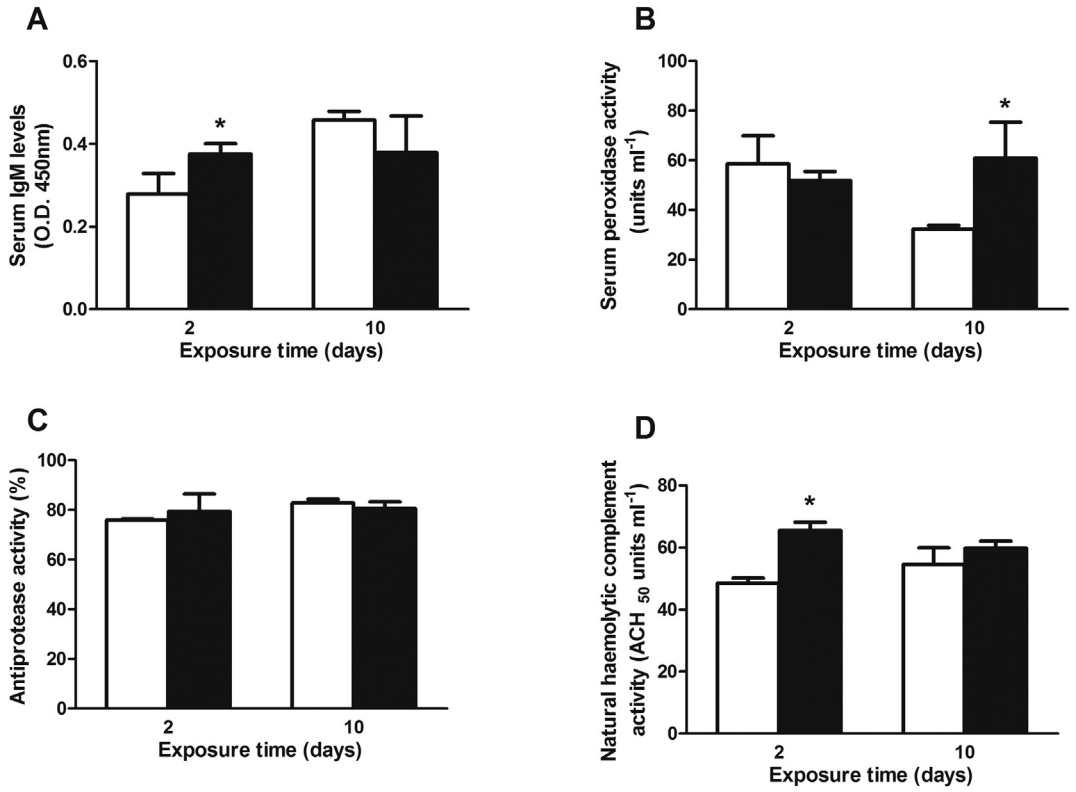

Fig. 4. Humoral immune response of European sea bass specimens unexposed (white bars) or exposed to 5 μM of As_2O_3 (black bars). Total IgM levels (OD at 450 nm) (A), serum peroxidase activity (units ml^{-1} of serum) (B), antiprotease activity (% in relation with the positive control) (C), and natural haemolytic complement activity (ACH_{50} units $ml^{-1}$of serum) (D). Bars are represented as mean ± SEM (n = 4). Asterisks denote significant differences between exposed and unexposed groups according to the Student *t*-test when $p < 0.05$.

2.7.6. Head-kidney and serum peroxidase activity

The peroxidase activity in serum or leucocytes was measured as previously (Quade and Roth, 1997). Briefly, 15 μl of serum were diluted with 135 μl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 50 μl of 20 mM TMB and 5 mM H_2O_2 were added. To determine the leucocyte peroxidase activity, 10^6 HK leucocytes in sRPMI were lysed with 50 μl (0.002%) of cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich) and, after centrifugation (400*g*, 10 min), 150 μl of the supernatants were transferred to fresh 96-well plates containing 25 μl of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour change reaction was stopped after 2 min by adding 50 μl of 2 M H_2SO_4 and the optical density was read at 450 nm in a plate reader. Standard samples without serum or leucocytes, respectively, were used as negative controls.

2.7.7. Phagocytosis assay

Phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by European sea bass head-kidney leukocytes was studied by flow cytometry (Rodríguez et al., 2003). Heat-killed and lyophilized yeast cells were labeled with fluorescein isothiocyanate (FITC; Sigma-Aldrich), washed and adjusted to 5×10^7 cells ml^{-1} of sRPMI. Samples consisted of 125 μl of labeled-yeast cells and 100 μl of HK leukocytes in sRPMI, afterwards, mixed, centrifuged (at 400*g* for 5 min at 22 °C), resuspended and incubated at 22 °C for 30 min. At the end of the incubation time, samples were placed on ice in order to stop phagocytosis process and 400 μl ice-cold PBS was added to dilute each sample. Extracellular fluorescence yeasts were quenched by adding 100 μl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labeled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay.

All samples were analyzed in a flow cytometer (Becton Dickinson FACSCalibur™) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10,000 events, which were acquired at a rate of 400 cells s^{-1}. Data were collected in the form of two-parameter side scatter (SSC) and forward scatter (FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerized system. Fluorescence histograms represented relative fluorescence on a logarithmic scale. Flow cytometer was set to analyze the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (FITC-cells) within the phagocytic cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from mean fluorescence intensity of phagocytic cells.

2.8. Statistical analysis

All samples were measured in triplicates and expressed as mean ± SEM. Data were statistically analyzed by Student *t*-test with SPSS v.19 (SPSS Inc., Chicago, USA). Differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Arsenic is accumulated in liver and gill, but not in skin

The bioaccumulation of As is one of the most important problems in fish, with trophic repercussions in human. Thus, the presence of As in gill, skin and liver tissues was evaluated, showing non-detected levels in all the unexposed specimens (Table 2). The present study revealed the presence of As in the liver at concentration of 582 ± 59 μg kg dry-weight^{-1} at 10 days whilst it was below the level of detection 2 days after As-exposure (Table 2). Surprisingly, As-accumulation was undetected in skin at any analyzed time. Finally, our results showed the highest levels of accumulation of As in gills with 1050 ± 121 μg kg dry-weight^{-1} and 1214 ± 119 μg kg dry-weight^{-1} at 2 and 10 days, respectively (Table 2).

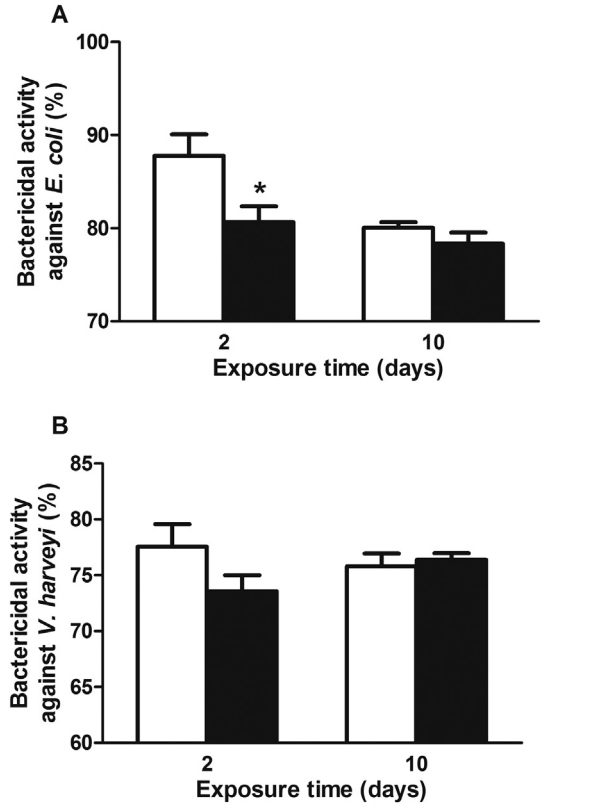

Fig. 5. Bactericidal activity (%) against *Escherichia coli* (A) and *Vibrio harveyi* (B) of serum from European sea bass specimens unexposed (white bars) or exposed to 5 μM of As_2O_3 (black bars). Bars are represented as mean ± SEM (n = 4). Asterisks denote significant differences between exposed and unexposed groups according to the Student *t*-test when $p < 0.05$.

3.2. As exposure clearly induces apoptosis cell death in liver from D. labrax

Liver is likely the most important tissue for detoxification of metalloids; however, gills and skin are in constant contact with the contaminated water. In the present study the liver showed a typical organization in unexposed fish (Fig. 1A) which is affected by As exposure at only 2 days of exposure showing cellular disorganization and apoptosis (Fig. 1B). At 10 days, the effects were more drastic with necrosis of hepatocytes, total disorganization of cellular structures and high degree of vacuolization (Fig. 1C). On the other hand, the skin of exposed fish showed no alterations at histological level (data not shown). Finally, the histological analysis revealed that gills were altered by As exposure showing hypertrophy and thickening as well as fusion of lamellae mainly at 2 but also at 10 days (Fig. 2B and C) comparing to the morphology of gills from unexposed fish (Fig. 2A).

The transcriptional levels of genes involved in apoptosis cell death such as B cell lymphoma 2-associated protein X (*bax*), B-cell lymphoma 2 (*bcl2*), caspase 3 (*casp3*), caspase 8 (*casp8*) and caspase 9 (*casp9*) were evaluated. The pro-apoptotic *bax* gene expression profile was significantly increased ($p = 0.043$) after As exposure at 10 days in liver of *D. labrax* (Fig. 3A). No changes were observed in transcript levels of skin and gill of exposed fish compared to the levels found on the same organs of unexposed fish (Fig. 3B and C). The anti-apoptotic *bcl2* was significantly down-regulated ($p = 0.039$) after As exposure at 2 days in liver of *D. labrax* (Fig. 3A). A significant up-regulation was observed in the executioner caspase, *casp3* ($p = 0.048$), as well as the two initiator caspases, *casp8* ($p = 0.013$) and *casp9* ($p = 0.035$) in the liver of *D. labrax* at 2 days after As exposure (Fig. 3A); however, the only caspase up-regulated was *casp9* ($p = 0.036$) at 2 days but not 10 days in gill of *D. labrax* after As exposure (Fig. 3C). Accordingly with the histological results, the gene expression profile in skin showed no changes at transcriptional level for the five analyzed genes involved in the apoptotic process (Fig. 3). Overall, the highest alterations of the gene expression pattern after waterborne As was observed in liver (mainly at 2 days) whilst both mucosal tissues skin and gill did not show great transcriptional changes under these conditions (Fig. 3).

3.3. Humoral immune responses in D. labrax are generally increased after As exposure

Five humoral immune activities were studied after As exposure at both 2 and 10 days (Fig. 4). First, total IgM levels in serum of *D. labrax* showed a significant increase at 2 days (but not at 10 days) after As exposure compared to the unexposed group (Fig. 4A). Secondly, serum peroxidase activity showed a significant increase but only at 10 days after As exposure (Fig. 4B). Regarding antiprotease activity, no changes were observed at both 2 and 10 days after As exposure (Fig. 4C). Natural haemolytic complement activity showed a significant increase in the exposed group after As waterborne compared to the control (unexposed) group at 2 days (Fig. 4D). Finally, bactericidal activity against both non-pathogenic and pathogenic bacteria (*E. coli* and *V. harveyi*, respectively) was tested (Fig. 5A and B, respectively). Our results showed a significant decrease in the bactericidal activity against *E. coli* at 2 days after As exposure compared to the unexposed group at the same time in serum of *D. labrax* (Fig. 5A).

3.4. Cellular immune response in D. labrax was decreased after As exposure

Among the cellular immune responses, respiratory burst and leucocyte peroxidase activities as well as phagocytic capacity and phagocytic ability were analyzed. Our results showed a partial decrease in some of the analyzed activities (Fig. 6). Respiratory burst activity was significantly decreased after As exposure at 2 and 10 days (Fig. 6A). In the case of leucocyte peroxidase activity, we only observed a significant decrease at 10 days (Fig. 6B). In relation with phagocytosis, both phagocytic capacity and ability (Fig. 6C and D, respectively) showed no significant decrease after As exposure.

4. Discussion

Metalloids are bioaccumulated and transferred through trophic chain (Uysal et al., 2008), and therefore represent a potential risk for human population as final consumers (Barak and Mason, 1990; Papagiannis et al., 2004; Yılmaz et al., 2007).

Different fish species have different susceptibility to metalloids, thus As has been tested in a range of concentrations between 0.5 and 100 μM as in previous studies (Banerjee et al., 2011; Datta et al., 2007, 2009a, 2009b; Hermann and Kim, 2005; Liao et al., 2004; Nayak et al., 2007). In addition, we have recently published that 5 μM of As_2O_3 is sub-lethal to gilthead seabream but produces important physiological changes (Guardiola et al., 2013). As regards to time, we have observed that the most significant changes take place at 2 and 10 days after exposure (Cordero et al., 2014; Guardiola et al., 2013; Guardiola et al., 2015) minimising or disappearing the effects after one month.

The accumulation of As in fish tissues may trigger important consequences not only for fish but also for fish consumers. We have previously reported that As is accumulated in liver but not in muscle of gilthead seabream (Guardiola et al., 2013). Our present results have demonstrated that As is also accumulated in gills and liver, whilst this metalloid, surprisingly, is not accumulated in the skin. Taking all together, our findings may suggest that As is first accumulated in gills, being likely one of the most important entry sites for As, later in liver whereas skin and muscle are not susceptible to accumulate As under these conditions.

In general, apoptosis is a programmed cell death process that occurs in complex organisms. At morphological levels, the appearance of cell shrinkage, pyknosis and karyorrhexis are the major changes in

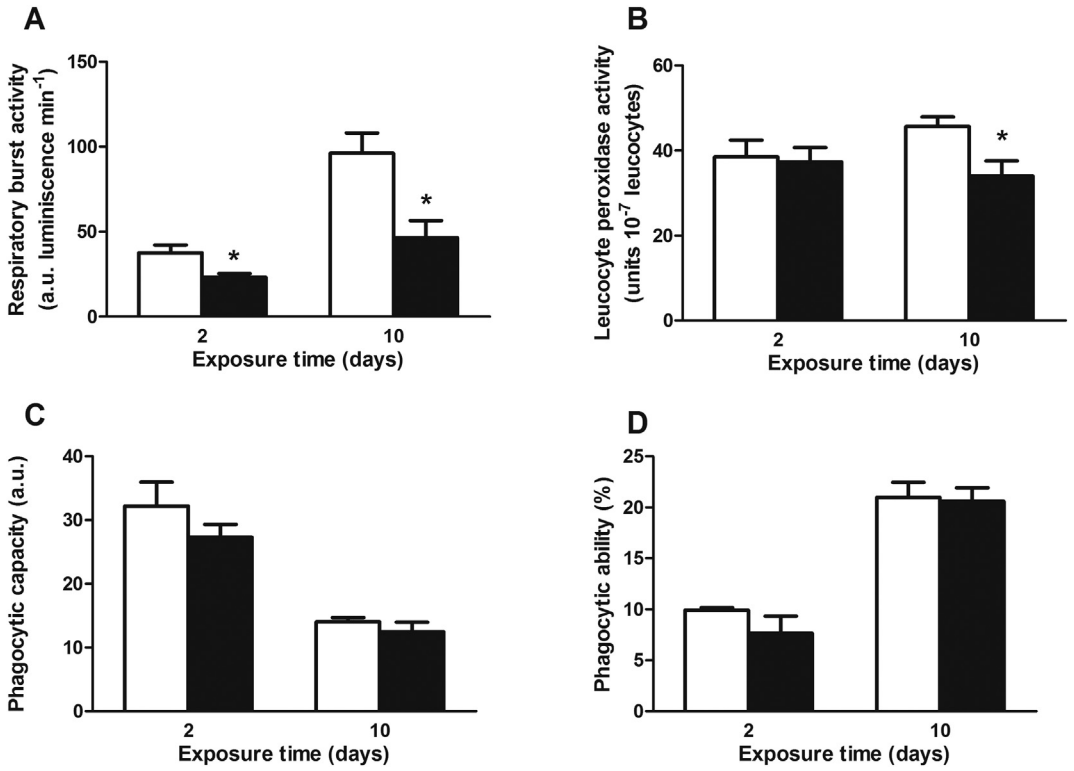

Fig. 6. Cellular immune response of European sea bass specimens unexposed (white bars) or exposed to 5 μM of As_2O_3 (black bars), evaluated through the respiratory burst activity [arbitrary units (a.u.) of luminescence min^{-1}] (A), leucocyte peroxidase activity (units 10^{-7} leucocytes) (B), phagocytic capacity [relative number of ingested yeasts per cell in arbitrary units (a.u.) from mean fluorescence intensity of phagocytic cells] (C) and phagocytic ability (% of cells with one or more ingested yeast cells within the phagocytic cell population) (D) of sea bass head-kidney leucocytes. Bars are represented as mean ± SEM (n = 4). Asterisks denote significant differences between exposed and unexposed groups according to the Student *t*-test when $p < 0.05$.

apoptotic processes (Elmore, 2007; Kerr et al., 1972). Furthermore, necrosis also includes cell swelling, formation of cytoplasmic vacuoles and karyolisis. Previous studies have reported cell swelling, hypertrophia and high degree of vacuolization in fish liver after As exposure (Chen et al., 2004; Guardiola et al., 2013; Shadat et al., 2016). The present study confirmed the presence of early apoptosis cell death at 2 days, which could be partially overlapped with necrosis after 10 days of As exposure since liver showed both morphological characteristics. In addition, apoptosis and necrosis can occur simultaneously depending on factors such as the intensity and duration of the stimulus (Zeiss, 2003). The protecting role against As waterborne of both gill and skin mucus deserves to be investigated since no evidences of apoptosis or necrosis have been observed in both tissues despite of lamellae hypertrophy was detected in gills. At molecular level, regulation of apoptosis is a very complex process. In the present work, at transcriptional level, the pro-apoptotic *bax* and anti-apoptotic *bcl2* regulators were up- and down-regulated by As exposure, respectively. Concomitantly, we showed the up-regulation of two initiator caspases (*casp8* and *casp9*) as well as the executioner *casp3* after 2 days of As exposure in liver. All this suggest that liver cells are suffering apoptosis as also evidenced by our histopathological data. In addition, both apoptosis and necrosis are induced after As_2O_3 exposure in fish cell lines (Morcillo et al., 2016; Selvaraj et al., 2013) and in the case of sea bass leucocytes (Morcillo et al., 2016 HKLs and PBLs) and DLB-1 cell line (Morcillo et al., 2016) we have found a positive relation between cell death by apoptosis and the transcription of *bcl2*, *bax* and/or *caspase* encoding genes. Therefore, we could speculate based on our data that: first, an early activation of apoptosis by the two main signaling pathways (intrinsic and extrinsic) after As exposure, due to the fact that the most significant changes at transcriptional level occurred after 2 days of exposition; and secondly, liver is the most susceptible target tissue to suffer apoptosis combined later with necrosis, in agreement with the morphological changes also observed in this study.

It is relatively well-known that metalloids exposure impairs the humoral response in fish (Cuesta et al., 2011; Ghosh et al., 2007). Nevertheless, little information is available regarding the main parameters that modulate both acquired and innate response at humoral levels. Our results revealed an increase in IgM levels, serum peroxidase and complement activities after As exposure in *D. labrax*. Similarly, an increase of Ig levels and peroxidase activity was detected in *Clarias batrachus* and *S. aurata*, respectively, after As exposure (Ghosh et al., 2007; Guardiola et al., 2013). However, no changes were observed in *S. aurata* complement activity (Guardiola et al., 2013) in a similar fashion than happens with the antiprotease activity after As exposure in the present research. The bactericidal activity is defined as the ability of humoral components to kill bacteria, which can be considered as a real indicator of the predisposition to infection. Thus, the observed decrease in serum bacterial activity after As exposure in *D. labrax* suggests that European sea bass specimens may be more susceptible to pathogenic infection after metalloid waterborne. In fact, it was previously reported that As induced the suppression in the bactericidal potential (Datta et al., 2009a). This point is also supported by another research, which showed As inhibit the ability to clear bacterial load in zebrafish (Nayak et al., 2007).

The cellular innate immune response, which mainly includes respiratory burst and myeloperoxidase activities, was partially inhibited by As exposure in the present study. These data are in agreement with previous studies that reported the inhibition of ROS over the time after As exposure in *D. rerio* (Hermann and Kim, 2005; Nayak et al., 2007). In addition, recent *in vitro* studies with As exposures revealed no changes in PMA-induced ROS production on European sea bass leucocytes (Morcillo et al., 2015a) whilst an increase of PMA-stimulated production of ROS was detected in gilthead seabream leucocytes (Morcillo et al., 2015b), suggesting different susceptibility to this metalloid

between these two important marine fish species. These data are supported by Guardiola et al. (2013) that reported an increase in the respiratory burst and peroxidase activities as well as the phagocytic ability of *S. aurata* after As exposure. Thus, based not only on the present research but also engaging our previous findings, we suggest that European sea bass and gilthead seabream have different immune signaling mechanisms on As toxicity or at least have different susceptibility to this metalloid.

Overall, the present paper revealed the effects on accumulation, histopathology and immunity in the marine teleost European sea bass caused by As_2O_3 exposure, a potent metalloid present naturally in the marine environment.

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