UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Toxicity Caused by Metals in Teleost Fish. Effects on Cellular Viability, Protection and Immunity

Toxicidad Producida por Metales en Peces Teleósteos. Efectos sobre la Viabilidad, Protección e Inmunidad Celular

Dña. Patricia Morcillo García

2016

To Whom It May Concern,

The PhD thesis entitled "Toxicity caused by metals in teleost fish. Effects on cellular viability, protection and immunity" is presented by Dr Patricia Morcillo Garcia and focused on *in vitro* effects of metals such as cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As) on fish cell lines or primary cell cultures derived from gilthead seabream (*Sparus aurata* L.) or European sea bass (*Dicentrarchus labrax* L.).

Particularly in the first part of the thesis a detailed investigation was shown about a characterization of a new European sea bass cell line (DLB-1) and the effects of metals on DLB-1 and SAF-1 (commercial from gilthead seabream) cell lines. This is the first study in which metal cytotoxicity has been evaluated in a fish brain cell line and results seem to support that the new cell line DLB-1 and the commercial SAF-1 cell lines are suitable for toxicological studies.

The second part is dedicated to the study of the effects of metals on primary cultures of seabream and sea bass erythrocytes, and head-kidney leucocytes (HKLs) and blood leucocytes (PBLs) including the evaluation of viability and gene expression alteration of oxidative stress, cell protection or cell death and immunotoxicity.

The Fish erythrocytes head-kidney and peripheral blood leucocytes incubated with each one of the metals exhibited alteration in gene expression profile of *mta*, *sod*, *cat*, *prx1*, *gr*, *hsp70* y *90*, *bax* and *calp1* indicating cellular protection, stress, oxidative stress and apoptosis death. Interestingly, the expression of immune-related genes was great altered by Hg, which included down-regulation of *ighm* and *hepc*, as well as the up-regulation of *il1b* mRNA levels. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish leucocytes *in vitro* and insight into the mechanisms at gene level and present substantial elements of originality; in fact Dr. Morcillo has made a fundamental contribution in the field of Fish immunology and biology.

Overall, the topic of the Dr. Morcillo PhD thesis is very appealing for its involvement in the scenario of aquaculture and immunotoxicology and, for this purpose, several advanced method and techniques belonging to immunology and physiology field have been exploited to elucidate the behavior of fish cells.

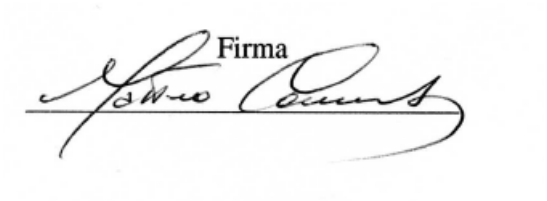
The PhD thesis is distinguished for the ability to combine theoretical and experimental analysis of the proposal phase and realization of the results achieved.

Notably, all the extensive research work performed during Dr Morcillo PhD thesis allowed the publication of 6 article on peer reviewed journals that could be considered an excellent performance for a PhD student.

Therefore, I strongly support to be awarded the PhD with "European Mention". I also invited to go ahead in his career of motivated researcher at University or for a competitive Company in this Field

Palermo May 25, 2016

Prof. Matteo Cammarata
Associate Professor in Zoology
President of Italian Association of Developmental and Comparative Immunobiology



UNIVERSITY OF ABERDEEN

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To Whom It May Concern,

PhD thesis: Toxicity caused by metals in teleost fish. Effects on cellular viability, protection and immunity

This work investigates the cytotoxic effects of heavy metals including Cd, Hg, Pb and on cells of gilthead seabream and European sea bass. The thesis contains General Introduction, 5 data chapters and general discussion. In Chapter 1, a permanent cell line (DLB-1) derived from brain was developed and characterised. The effects of different metals on the DLB-1 cells were comparatively studied. The results indicate that the DLB-1 cells are responsive to exposure of metals. It has been concluded that the cell line is useful for toxicological research in fish. In Chapter 2, similar approaches described in Chapter 1 were applied to evaluate the toxicity, oxidative stress and apoptosis pathway in a seabream cell line SAF-1 following treatment of heavy metals. It has been shown that exposure of SAF-1 cells to heavy metals resulted in increased production of the ROS species and induction of cell apoptosis. This observation was also supported by the data of the gene expression analyses. Chapter 3 investigates the effects of metals on fish erythrocytes isolated from gilthead seabream and European sea bass. Viability of the erythrocytes was assessed after exposure to metals. Expression of several key genes involved in cellular protection, stress and apoptosis were analysed. Chapters 4-6 analysed the in vitro effects of metals on the primary leucocytes

isolated from immune organs (head kidney and blood) for the gilthead seabream and European sea bass. In both species, exposure to Cd, MeHg, Pb or As reduced the viability of leucocytes in a dose dependent manner. Interestingly, head kidney leucocytes were shown to be more sensitive to the metals than blood leucocytes. At the molecular level, genes such as bcl2 and casp3, known to be activated during apoptosis, were induced. Strikingly, genes involved in immune responses such as il1b and ighm were suppressed.

Overall, the quality of the science is of high standard as exemplified by the number of papers published. More importantly, a range of cellular and molecular assays have been developed for evaluating the cytotoxicity of metals/metal pollutants in fish using the cell culture system. These tools will be invaluable for future research to assess the impacts of the metal pollutants on farmed fish and aquatic animals in general. In particular, the DLB-1 cell line will have long term benefits for fish biologists.

In summary, the thesis is well structured and well written with clear aims and discussions. The experiments are hypothesis-driven and logically designed. Data are adequately analysed and interpretation of the results is scientifically sound. In no doubt, I strongly recommend the thesis to be awarded a Degree of Doctor of Philosophy with International Mention.

Dr Jun Zou

Senior Research Fellow

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University of Aberdeen

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Publications/collaborations related to the Thesis:

1. **Morcillo, P.,** Cordero, H., Meseguer, J., Esteban, M.A., Cuesta, A., 2015. Toxicological *in vitro* effects of heavy metals on gilthead seabream (*Sparus aurata* L.) head-kidney leucocytes. Toxicology In Vitro, 30, 412-420.

2. **Morcillo, P**., Cordero, H., Meseguer, J., Esteban, M.A., Cuesta, A., 2015. *In vitro* immunotoxicological effects of heavy metals on European sea bass (*Dicentrarchus labrax* L.) head-kidney leucocytes. Fish and Sellfish Immunology, 47, 245-254.

3. **Morcillo, P**., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the gilthead seabream (*Sparus aurata* L.) cell line SAF-1. Chemeosphere, 144, 225-233.

4. **Morcillo, P.**, Romero, D., Meseguer, J., Esteban, M.A., Cuesta, A., 2016. Cytotoxicity and alterations at transcriptional level caused by metals on fish erythrocytes *in vitro*. Environmental Science Pollution Research, doi: 10.1007/s11356-016-6445-3.

5. **Morcillo, P**., Meseguer, J., Esteban, M.A., Cuesta, A., 2016. *In vitro* effects of metals on isolated head-kidney and blood leucocytes of the teleost fish *Sparus aurata* L. and *Dicentrarchus labrax* L. Fish and Sellfish Immunology, 54, 77-85.

6**. Morcillo, P.,** Chaves-Pozo, E., Meseguer, J., Esteban, M.A., Cuesta, A., 2016. Establishment of a fish brain cell line (DLB-1) and metal neurotoxicology. Neurotoxicology (submitted).

Other publications:

7. Trapani, A., Mandracchia, D., Di Franco, C., Cordero, H., **Morcillo, P.**, Comparelli, R., Cuesta, A., Esteban, M.A., 2015. *In vitro* characterization of 6-Coumarin loaded solid lipid nanoparticles and their uptake by immunocompetent fish cells. Colloids and Surfaces B: Biointerfaces, 127, 79-88.

8. Valero, Y., **Morcillo, P.,** Meseguer, J., Buonocore, F., Esteban, M.A., Chaves-Pozo, E., Cuesta, A., 2015. Characterization of the IFN pathway in the teleost fish gonad against vertically transmitted viral nervous necrosis virus. Journal of General Virology, 96, 2176-2187.

9. Cordero, H., **Morcillo, P**., Cuesta, A., Brinchmann, M.F., Esteban, M.A., 2015. Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. Journal of Proteomics, 132, 41-50.

10. Cordero, H., **Morcillo, P.**, Meseguer, J., Cuesta, A., Esteban, M.A., 2016. Effects of *Shewanella putrefaciens* on innate immunity and cytokine expression profile upon high stocking density of gilthead seabream specimens. Fish and Shellfish Immunology, 51, 33-40.

18 International communications, which the most important are:

1. **Morcillo, P**., Esteban, M.A., Cuesta, A.
Title: Cytotoxic effects of methylmercury and arsenic in the gilthead seabream cell line SAF-1.
11th International Congress on the Biology of Fish.
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City of event: Edinburgh, Scottland.

2. **Morcillo, P**., Olveira, P.J., Bandín, I., Esteban, M.A., Meseguer, J., Chaves-Pozo, E., Cuesta, A.
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Title: Usefulness of fish head-kidney leucocytes to assess the immunotoxicological effects of contaminants.

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Preface

This dissertation is submitted in fulfilment of the requirements for the degree of *Philosophiae Doctor* (PhD) at the Faculty of Biology, University of Murcia (UMU), Spain. The different studies compiled in this dissertation represent original research carried out over a period of around four years, as part of some projects and grants:

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The present dissertation included the following persons:

Alberto Cuesta, PhD, Lecturer (UMU), primary supervisor.

María Ángeles Esteban, PhD, Lecturer (UMU), co-supervisor.

José Meseguer, PhD, professor (UMU), research group leader.

Héctor Cordero, PhD student (UMU).

Diego Romero, PhD, Lecturer (UMU).

Elena Chaves-Pozo, PhD, researcher of the IEO (Spanish Institute of Oceanography).

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Seguramente hay un rumbo posiblemente y de

muchas maneras personal y único.

Posiblemente haya un rumbo seguramente y de muchas maneras el mismo para todos.

Hay un rumbo seguro y de alguna manera posible.

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cuyas ausencias me han enseñado a salir del fondo del mar.

Querido Mano,

esta es la carta que tantas veces pensé en escribirte pero nunca tuve el valor de hacerlo, quizás por miedo, quizás porque nunca me quise imaginar una vida sin tí.

Quiero que sepas que has sido y eres mi mayor referente. Me enseñaste a luchar por lo que creía, a no tirar la toalla, a tener mi propia opinión de las cosas, a no quedarme de brazos cruzados ante las injusticias y a pelear por mis derechos, aunque eso me haya acarreado muchos problemas en esta sociedad en la que vivimos. Como tú bien me decías, no hay nada como acostarse sin ningún remordimiento, sabiendo que has hecho lo correcto en cada momento.

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Por desgracia, también me enseñaste que la vida no es más que un soplo de aire que se va, que hay que vivirla en cada instante, porque nunca se sabe que puede pasar, y que la muerte no engaña, te señala con el dedo, te dispara, y adiós.

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Siempre tu “Patri”

Mamá, por qué dejaste que me hiciera mayor,

por qué no me hablas como cuando era niño,
por qué no me consuelas cuando lloro,
como cuando me pegaban los otros niños.

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y fundo el plomo en la “fridera” vieja,
y tú me haces pan torrao
con aceite y azúcar.

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como aquel que a tantas enamore,
por qué no me cuentas la vida
de tanta y tanta gente.

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la Placida, la señora Antonia
o cualquiera otra de la calle La Vía.

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a las nueve en raya y a la opia y muda.

Mamá, por que quise hacer la revolución,
arreglar el mundo, desterrar la injusticia,
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que el sol es solo sol si brilla en tí,
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delante de nuestros ojos.

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que valga por todos los que no te di,
y pedirte perdón por no haber sabido
hacer mi sueño realidad,
como hubiese querido.

Para mi madre,
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“Érase una vez, un lobito bueno
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una bruja hermosa
y un pirata honrado.
Todas esta cosas había una vez,
cuando yo soñaba un mundo al revés“.

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Index

Abbreviations

ABC	ATP-binding cassette
AESAN	Spanish Food Safety and Nutrition Agency
AIF	Apoptosis-inducing factor
Al	Aluminium
ALAD	Delta-aminolevulinic acid dehydratase
AMPs	Antimicrobial peptides
Apaf-1	Apoptotic activating factor-1
AQPs	Aquaglyceroporins
As	Arsenic
ASBB	Asian sea bass brain cell line
At	Astatine
ATOX1	Antioxidant 1 copper chaperone
ATP	Adenosine triphosphate
ATP7A	P-type ATPase
B	Boron
BAX	Bcl-2 associated X protein
BB	Barramundi brain cell line
BCL2	B-cell lymphoma 2
BF-2	Bluegill fry cell line
Ca	Calcium
CADD	Computer aided drug design
CALP1	Calpain1
CASP3	Caspase-3
CAT	Catalase
CB	Catla brain cell line
CC	Chemokines
Cd	Cadmium
cDNA	Complementary DNA
CHSE-124	Chinook salmon embryonic cell line
Cl^-	Chloride ion
CMV	Human cytomegalovirus promoter
Co	Cobalt
CORO1A	Coronin-1A
CPB	Chinese perch brain cell line
Cr	Chromium
CRF-1	Clonal red sea bream fin-1 cell line
CSFs	Colony stimulating factors
CSH	*Cynoglossus semilaevis* heart cell line
CTR1	Copper transporter 1
Cu	Copper

CuZnSOD	Copper/zinc SOD
CV	Crystal violet
CYP450	Cytochrome P450
DHR	Dihydrorhodamine 123
DLB-1	*Dicentrarchus labrax* brain 1
DLEC	*Dicentrarchus labrax* embryonic cell line
DMT1	Divalent metal transport 1
DNA	Deoxyribonucleic acid
EC_{50}	Effective concentration 50
ECSOD	Extracellular SOD
EDTA	Ethylenediaminetetraacetic acid
eelB	American eel brain cell line
EF1α	Elongation factor 1-alpha
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPC	Epithelioma papulosum cyprinid cell line
FADD	Fas-associated death domain protein
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal bovine serum
FDA	Fluorescein diacetate
Fe	Iron
FG9307	Flounder gill cell line
FHM	Fathead minnow cell line
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G	Granulocytes
G0 phase	Gap 0 cell cycle
G1 phase	Gap 1 cell cycle
G2 phase	Gap 2 cell cycle
GALT	Gut-associated lymphoid tissue
GB	Grouper brain cell line
GF-1	Grouper fin cell line
GFAP	Glial fibrillary acidic protein
GFM	Goldfish muscle cell line
GFP	Green fluorescent protein
GLUT	Glucose transporters
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide

H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HbO_2	Oxyhemoglobin
HBSS	Hank's buffered salt solution
HE	Haematoxylin-eosin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HKLs	Head-kidney leucocytes
HOCl	Hypochlorous acid
HSP	Heat-shock protein
IARC	International Agency for Research in Cancer
IEO	Instituto Español de Oceanografía
IFN	Interferon
Ig	Immunoglobulins
IGHM	Immunoglobulin M heavy chain
IL	Interleukin
L-15	Leibowitz medium
LDH	Lactate dehydrogenase
LOOH	Lipid hydroperoxide
MAF	Macrophage-activating factors
MAP2	Microtubule-associated protein 2
MDR	Multidrug resistance
Hg	Mercury
MeHg	Methylmercury
Mg	Magnesium
MG-3	Mrigal gill cell line
MHC	Major histocompatibility complex
MIFs	Migration inhibitory factors
MM	Monocyte-macrophage
Mn	Manganese
MnSOD	Manganese SOD
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRPs	Multidrug-resistance-associated proteins
M phase	Mitosis phase cell cycle
MT	Metallothionein
MTT	(3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide)
Mx	Myxovirus resistance proteins Interleukin 1 beta
NADPH	Nicotinamide adenine dinucleotide phosphate
NCC	Non-specific cytotoxic cells
Nec-1	Inhibitor necrostatin-1

NF	Neurofilament
Ni	Nickel
NK	Natural killer
NO•	Nitric oxide
NR	Neutral red
$O2^{\bullet-}$	Superoxide radical
OH•	Hydroxyl radical
ONOO-	Peroxynitrite
P	Cellular passage
p53	Tumor protein p53
PAHs	Polycyclic aromatic hydrocarbons
PARP-1	Poly (ADP-ribose) polymerase 1
Pb	Lead
PBLs	Peripheral blood leucocytes
PBS	Phosphate-buffered saline
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
Pgps	P-glycoproteins
PHOX22	NADPH oxidase, subunit Phox22
PHOX40	NADPH oxidase, subunit Phox40
PI	Propidium iodide
pKa	Negative logarithmic of the acid dissociation constant
PLHC-1	*Poeciliopsis lucida* hepatocellular carcinoma line 1
PMA	Phorbol myristate acetate
POBC	*Paralichthys olivaceus* brain cell line
ppm	Parts per million
PRX1	Peroxiredoxin 1
PRX2	Peroxiredoxin 2
PTP	Mitochondrial permeability transition pore
PTPC	Permeability transition pore complex
PUFAs	Polyunsaturated fatty acids
RBFOX3	RNA Binding Protein, Fox-1 Homolog (*C. Elegans*) 3
RG-1	Rohu gill cell line
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RNS	Reactive nitrogen species
ROO•	Lipid peroxyl radical
ROS	Reactive oxygen species
RTG-2	Rainbow trout gonadal cell line
SAA	Serum amyloid A
S phase	DNA synthesis cell cycle

SAF-1	*Sparus aurata* fibroblast 1
SARs	Structure activity relationship
SBB-W1	European sea bass brain cell line
Se	Selenium
SE	Standard error
SEM	Scanning electron microscopy
Sn	Tin
SnRV	Snakehead retrovirus
SOD	Cu/Zn superoxide dismutase
SPH	Sea perch heart cell line
RPMI	RPMI-1640 culture medium
SSC	Side scatter
SV40	Simian virus-40
Tc	Cytotoxic T lymphocyte
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCPBP	Trout C-polysaccharide binding protein
TCRB	T cell receptor beta chain
TERT	Telomerase reverse transcriptase protein
TGFs	Transforming growth factors
Th	Thymus
Thy	Helper T lymphocyte
TNFR	Tumor necrosis factor receptor
TNFs	Tumor necrosis factors
WSF	White sturgeon fin cell line
ZC-7901	Grass carp lip cell line
ZFL	Zebrafish liver cell line
ZIP	Zinc transporters
Zn	Zinc

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Summary

During the present Doctoral Thesis, we have studied the *in vitro* effects of metals such as cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As) on fish cell lines or primary cell cultures derived from gilthead seabream (*Sparus aurata* L.) or European sea bass (*Dicentrarchus labrax* L.) specimens, which are two marine fish species of great importance in aquaculture. Thus, the first part includes **two chapters** devoted to the establishment and characterization of a new European sea bass cell line (DLB-1) and the effects of metals on DLB-1 and SAF-1 (from gilthead seabream) cell lines. The second part is divided in **4 chapters** dedicated to the study of the effects of metals on primary cultures of seabream and sea bass erythrocytes (**chapter 3**) and head-kidney leucocytes (HKLs) and blood leucocytes (PBLs) (**chapter 4**). In all cases, parameters such as viability and alteration in the gene expression profile related to oxidative stress, cell protection or cell death have been evaluated. In addition, when leucocytes have been used, immunotoxicity has also been evaluated in the **chapters 5** and **6**.

1. In the first **chapter**, we have established and characterized a new cell line derived from the European sea bass brain, called DLB-1. Indeed, no commercial cell lines from sea bass are available. Furthermore, its usefulness in toxicology studies has been tested. DLB-1 cells showed a rapid cell division, glial origin and low rate of transfection. When DLB-1 cells were exposed to different metals (As, Pb, MeHg or Cd) for 24 h the cells resulted killed in a dose-dependent manner. Neutral red (NR) uptake was the most sensitive colorimetric technique and Cd was the most toxic metal (EC_{50}=0.004 mM) followed by MeHg, As and Pb. Moreover, Cd and Pb induced the greatest production of ROS. Interestingly, cell cycle analysis of DLB-1 cells exposed to metals showed that exposure to Cd, MeHg or Pb significantly increased the percentage of cells in interphase G0/G1 cells whereas Pb and As increased the percentage of cells in S phase and almost abrogated the G2/M phase. Moreover, all the metals induce apoptosis cell death as indicated by sub-Go/G1 population in the cell cycle analysis and annexin binding. Finally, exposure of DLB-1 cells to metals also produce significant alterations at gene expression level that confirm the above results such as down-regulation of anti-apoptotic (*bcl2*) and antioxidant system (*sod* and *cat*) gene transcription. This is the first

study in which metal cytotoxicity has been evaluated in a fish brain cell line and results seem to support that DLB-1 cells are suitable for toxicological studies.

2. Similarly, in the second **chapter**, the cytotoxic effects of metals Cd, MeHg, Pb and

As after 24 h exposure on the commercial SAF-1 cell line derived from fins of gilthead seabream were evaluated. NR, MTT-tetrazolio (MTT), crystal violet (CV) and lactate dehydrogenase (LDH) viability tests showed that SAF-1 cells exposed to the above metals produced a dose-dependent reduction in the number of viable cells. MeHg showed the highest toxicity (EC_{50}=0.01 mM) followed by As, Cd and Pb. NR was the most sensitive method followed by MTT, CV and LDH. SAF-1 cells incubated with each one of the metals also exhibited an increase in the production of ROS and apoptosis cell death. Moreover, the corresponding gene expression profiles pointed to the induction of the metallothionein protective system, cellular and oxidative stress and apoptosis after metal exposure for 24 h. This report describes and compares tools for evaluating the potential effects of marine contamination using the SAF-1 cell line.

3. The aim of the third **chapter** was to evaluate the cytotoxicity of metals Cd, Hg, Pb

and As on circulating erythrocytes from marine gilthead seabream and European sea bass specimens. Exposure to metals produced a dose-dependent reduction in the viability, and Hg showed the highest toxicity (EC_{50}=0.0024mM) followed by Cd, As and Pb. Moreover, fish erythrocytes incubated with each one of the metals exhibited alteration in gene expression profile of *mta*, *sod*, *cat*, *prx1*, *gr*, *hsp70* y *90*, *bax* and *calp1* indicating cellular protection, stress, oxidative stress and apoptosis death. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish erythrocytes *in vitro*.

4. In the fourth **chapter**, head-kidney and peripheral blood leucocytes (HKLs and PBLs, respectively) from gilthead seabream and European sea bass specimens were exposed for 24 h to Cd, MeHg, Pb or As being evaluated the resulting cytotoxicity. Exposure to metals produced a dose-dependent reduction in the viability, and MeHg showed the highest toxicity followed by Cd, As and Pb. Interestingly, leucocytes from European sea bass are more resistant to metal exposure than those from gilthead seabream. Similarly, HKLs are always more sensitive than those isolated from blood from the same fish species. Thus, genes related to cellular protection (*mta, hsp*) and oxidative stress (*sod, cat, gr*) were, in general, down-regulated in seabream HKLs but up-regulated in seabream PBLs and sea bass HKLs and PBLs. In addition, this profile lead to the increase of expression in genes related to apoptosis (*bax, casp3*). Finally, transcription of genes involved in immunity (*il1b, ighm*) was down-regulated, mainly in seabream leucocytes. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish leucocytes *in vitro* and insight into the mechanisms at gene level.

5. In the fifth **chapter**, the effects of metals (Cd, Hg, Pb or As) on viability, oxidative stress and innate immune parameters of isolated HKLs from gilthead seabream were studied. Cytotoxicity results indicated that short exposures (30 min or 2 h) to Hg promoted both apoptosis and necrosis cell death of leucocytes whilst Cd, Pb and As did only by apoptosis, in all cases in a concentration- and time-dependent manner. In addition, production of ROS was induced by Cd, Hg and As metals. Cd failed to change phagocytosis but Hg and As increased the percentage of phagocytic cells but decreased the number of ingested particles per cell whilst Pb increased both phagocytic parameters. On the other hand, respiratory burst activity was significantly reduced by incubation with Cd, Hg and As but increased with Pb. Furthermore, the gene expression profiles partly support the functional finding of this work. This study provides an *in vitro* approach for elucidating the metals toxicity, and particularly the immunotoxicity, in fish leucocytes.

6. In the sixth and last **chapter** of this Thesis, HKLs from European sea bass were exposed to metals Cd, Hg, As and Pb for 30 min or 2 h. Production of ROS was induced by Cd, Hg and As, mainly after 30 min of exposure. Cd and Hg promoted both apoptosis and necrosis cell death while Pb and As did only by apoptosis, in all cases in a concentration-dependent manner. Moreover, expression of genes related to oxidative stress and apoptosis were significantly induced by Hg and Pb but down-regulated by As. In addition, the expression of *mta* and h*sp70* genes was up-regulated by Cd and Pb exposure though this transcript and down-regulated by Hg. Cd, MeHg and As reduced the phagocytic ability but Pb increased it. Interestingly, all the metals decreased the phagocytic capacity. Leucocyte respiratory burst changed depending on the metal exposure, usually in a time- and dose-manner. Interestingly, the expression of immune-related genes was great altered by Hg, which included down-regulation of *ighm* and *hepc*, as well as the up-regulation of *il1b* mRNA levels.

Introduction

1. Overview

Toxicology is the scientific field which evaluate the adverse effects of chemicals and the area of Ecotoxicology is devoted to study the effects of chemical pollutants on ecosystems and their impact on the populations and communities. Contamination is one of the major problems associated with the environmental sciences. Over the last decades the levels of pollutants on the aquatic environment have increased as a result of mining, forestry, waste disposal and fuel combustion. Pollutants, or ecotoxicants, could be divided according to different criteria (Austin, 1999; Bols et al., 2001). Thus, based on the biological function; they can be classified as insecticides, herbicides, fungicides and environmental estrogens. However, they are more known by its chemical structure and are divided in polycyclic aromatic hydrocarbons (PAHs), dioxins and furans, polychlorinated biphenyls (PCBs), phenols, organometallics and metals. In the present Thesis, we will focus on the effects of metals on fish because they are persistent in the environment and can be bioaccumulated and biomagnified along the food chain (Castro-González and Méndez-Armenta, 2008; Mendil et al., 2010). In addition, they might be a problem not only for fish living there but also for human consumption.

Numerous toxicological studies have gained increasing interest in order to understand the impact of metals on aquatic communities, which are particularly vulnerable. Marine invertebrates such as molluscs and crustaceans have been used to assess pollution (Lagadic and Caquet, 1998), but a more attractive group are fish, which represent the most diverse group of vertebrates (Cossins and Crawford, 2005) and have genetic relatedness to the higher vertebrates including mammals. It is widely known that fish are a great source of metals (Hg, Al, Se, Cd, Pb, As, Cu, Cr, Fe, Mn, Mb, Va and Zn) in our food and their accumulation could represent a serious risk for human beings (EFSA, 2012; Minganti et al., 2010). Although fish have always been perceived as a healthy and nutritive food (Serra-Majem et al., 2007), a recent report of the Spanish Food Safety and Nutrition Agency (AESAN) has raised public concern as it claimed that the levels of some metals (mainly Hg and Cd) in certain fish species make them unsuitable or be restricted for children and pregnant women consumption (AESAN, 2011). According to the Environmental Protection Agency (EPA), the content of Hg in some fish species such as Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*) is less

than 0.09 ppm (parts per million), in European sea bass (*Dicentrarchus labrax*) from 0.3 to 0.49 ppm and in tuna (several fish species belonging to the *Thunnini* tribe) or swordfish (*Xiphias gladius*) more than 5 ppm. These data are worrying us if we consider that the projections show an increase in the demand for seafood products to year 2,030. In Europe, the average *per capita* consumption will increase from 22 kg/person/year in 1,998 to 24 kg/person/year in 2,030. The two additional kg per capita signifies that the net supply will have to increase by 1.6 million tonnes (Mt) (respectively, 1.1 Mt for the 2 extra kilos per person and 550,000 tonnes due to the 22 million population growth over the period).

In this regard, aquaculture will be one of the fields in the coming years that will increase significantly, trying to compensate this negative balance for human consumption. According to the FAO (Food and Agriculture Organization of the United Nations), extractive fishing only covers 60% of the annual world fish production (FAO, 2012), a situation in which aquaculture is seen as the only way to satisfy the demand in the near future. Concretely, Spain is the third member state of the European Union (EU) with a high production of fish from aquaculture as well as in the production of gilthead seabream (*Sparus aurata*) (16,795 tonnes in 2,013) and European sea bass (14,707 tonnes in 2,013) emerging as an area of economic activity of great strategic importance. Furthermore, it is located in the 20th position in the world ranking of aquaculture producers and 9th in the world ranking of fish exporters. Therefore, farmers have to know and control the impact of the environmental contaminants in the species produced for humans (Cuesta et al., 2011). In this specific field, relevant fish species for aquaculture are not commonly used as models in ecotoxicological studies and very few have used marine cell lines or primary cell cultures to screen and evaluate toxicity in *in vitro* conditions. The use of cell cultures in fish has been proposed as an alternative method to animal experimentation. In fact, *in vitro* fish cell assays are able to generate comparable results on relative potency ranking and effect of toxicants to the *in vivo* acute lethality test (Segner, 2004) and at the same time allow better mechanistic characterization. Although some studies have evaluated certain aspects of gilthead seabream and European sea bass biology after exposure to metals *in vivo* (García-Santos et al., 2011; Guardiola et al., 2013a, b; Guardiola et al., 2016; Roche and Bogé, 2000; Roméo et al., 2000; Souid et al., 2013), very few studies have dealt with this aspect

under *in vitro* conditions (Bogé and Roche, 2004; Minghetti et al., 2011; Vazzana et al., 2014).

Therefore, knowledge of the toxicological effects of pollutants, and specially metals, on fish and of the species of interest in aquaculture, in particular, has become one of the primary objectives in research applied to aquaculture. Furthermore, the use of alternative methods to animal testing has gained great interest in the field of toxicology. In this context, the aim of this Thesis was to assess the cytotoxicity effects of four metals (Cd, Hg, As and Pb) on cell lines and primary cell cultures from gilthead seabream and European sea bass, the most important cultured fish species in the Mediterranean area.

2. Toxicology by metals in fish

Toxicity in fish is the culmination of a series of events involving various physical, chemical, and biological processes (Erickson et al., 2008). Pollutants are released to the environment from different sources; enter aquatic systems by effluents, atmospheric deposition, runoff, and groundwater; and become distributed throughout the water column and underlying sediment. Aquatic organisms become contaminated via contact with water or sediment and via their own food. Fish represents a group of vertebrates ecologically and economically important which play a role in the food chain as consumers (predators) and consumed (prey). Fish accumulate pollutants both by ingestion of contaminated food and by contact of their surfaces (skin, gills and digestive tract) with contaminated material (Van der Oost et al., 2003). Accumulated chemicals are distributed throughout the fish, and some of these chemicals reach a site of action to elicit toxic effects. Thus, the accumulation of toxics may have a negative impact not only for fish but also for their consumers (Fernandes et al., 2008). Therefore, fish occupy a prominent position in the field of toxicology; they constitute a relevant group of test organisms to evaluate the biological effects of toxic chemicals. Knowledge and understanding how toxics can affect cultured fish as well as the levels they might transfer to humans is important information for the aquaculture industry to develop efficient restrain measures.

2.1. Metal definition and classification

Nowadays, in toxicology, the term metal substitutes the previous designation of heavy metals. Heavy metals were considered any toxic metal and this include some metals, metalloids, transition metals, basic metals, lanthanides and actinides (Duffus, 2002). Metals are defined as relatively dense chemical elements causing toxic effects and differ from other toxic substances in that they are neither created nor destroyed by humans. About 80 of the 105 elements in the periodic table are regarded as metals. Metalloids occur along the diagonal line through the p block from boron (B) to astatine (At). Elements in the upper right of this line display increasing non-metallic behaviour; elements in the lower left display increasing metallic behaviour. Metals are elements with an atomic weight greater than 20 and characterised by similar atomic electron configuration in the outer orbitals and can be categorized as biologically essential and nonessential (Goyer and Clarkson, 1996). Nonessential metals such as tin (Sn), aluminium (Al), Cd, Hg, and Pb have no demonstrated biological function (also called xenobiotics or foreign elements) and its toxicity rises with increasing concentrations in a similar way to what happen with the metalloid As. Essential metals, for example copper (Cu), zinc (Zn), cobalt (Co), nickel (Ni), selenium (Se) and iron (Fe) are those with a biological function (Kennedy, 2011) though they can be toxic at high or non-biological concentrations.

Human activities such as mining, industry and sewage treatment discharges as well as electronic wastes (computers, printers, photocopy machines, TV sets, mobile phones and toys) and agriculture (agriculture fertilisers) are some examples of anthropogenic sources contributing to the elevated levels of trace metals. Metal pollution can harm aquatic organisms through lethal and sublethal effects and can reduce or eliminate species from an ecosystem through increased susceptibility to fish disease, mortality and decreased fecundity (Kibria, 2014). Indeed, fish can bioaccumulated metals to several orders of magnitude (thousands to million times) above background levels in the aquatic environment (EFSA, 2012; Minganti et al., 2010).

Distribution of metals in water is determined by the physico-chemical properties of the metal and it is influenced by complex and diverse environmental parameters including hydrophobicity, pH, salinity, hardness (Ca^{2+} and Mg^{2+} concentration) and interaction of metals with biotic and abiotic ligands (Erickson et al., 2008). For example, the

bioavailability (ability of a metal to bind or traverse/cross the cell membrane) of Cu decrease with increasing hardness, with Ca^{2+} and or Mg^{+} competing with metals for surface binding sites on cell membranes (Komjarova and Blust, 2009). Moreover, a decrease in pH may increase the bioavailability of metals, resulting in metal desorption from colloidal and particulate matter, and dissociate some inorganic and organic metal complexes (Niyogi and Wood, 2004). Soluble metals include simple dissolved metal ions, metal ion complexes with organic ligands and with inorganic anions. For dissolved metals, there is considerable evidence that the dissolved ionic form is the major bioavailable form and correlates best with metal toxicity. Metals can also be found in organic forms as bioavailable organometallic compounds such as MeHg (Kibria, 2014). An important aspect of this chain of events is chemical accumulation and toxicity that depend not only on total chemical concentration in the environment but also on how readily the fish can absorb these different chemical species at the gill, across the skin, and within the digestive tract and on how chemical speciation affects distribution throughout the organism (Erickson et al., 2008).

2.2. Toxicokinetics: absorption, distribution and excretion

The processes of absorption, distribution, biotransformation, and excretion also determine the extent to which xenobiotics bioconcentrate and bioaccumulate in fish. Bioconcentration refers to the uptake and accumulation of chemicals directly from water. Bioaccumulation is a more inclusive term that describes chemical uptake and accumulation from all sources: water, diet, and sediments. Bioconcentration and bioaccumulation, particularly of persistent compounds, serve as indicators of past exposure for the animal; as indicators of potential exposure for higher trophic levels, including the human consumer; and as markers of potential toxicity.

In fish, metal absorption and distribution occurs as follows: first, metals cross the epithelium; second, they incorporate into blood including binding to plasma proteins, transport via the systemic circulation of freely dissolved and plasma-bound chemical to various tissues; and finally, they are transported from blood into tissues. Gills, digestive system, and, to a lesser extent, the skin, are the major sites of metal uptake in fish (Erickson et al., 2008). For crossing the cell membrane, there are three main processes for metal uptake: passive diffusion, channel penetration and endocytosis (Figure I). If the molecules are not charged they are able to pass directly through the cell membrane.

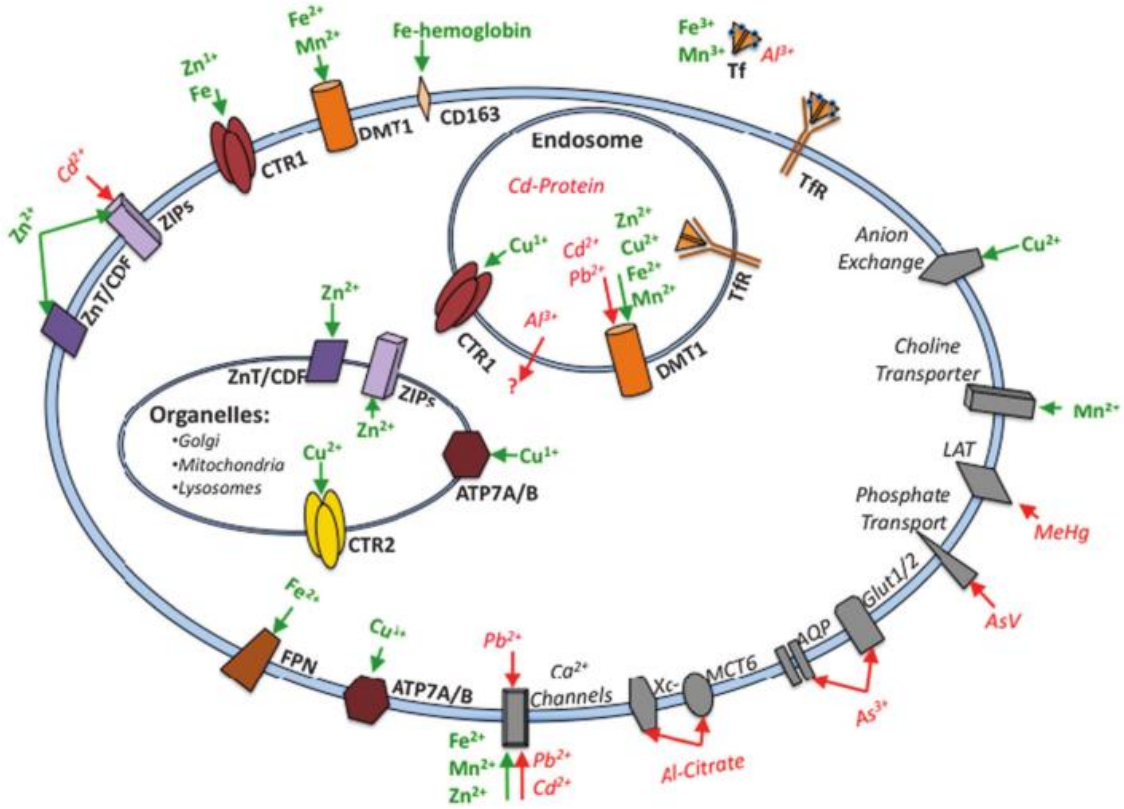

Figure I. Cellular transport mechanisms for essential and nonessential metals. Transporters whose primary functions are to transport metals are bolded, while those whose primary function is not the transport of metals are italicized. Essential metals are represented in green and non-essential metals in red (Ebany et al., 2012).

For instance, MeHg, due to its high lipophilicity, may diffuse directly across the membrane (Boudou et al., 1991; Huang et al., 2012). However, most hydrophilic metals with the correct size and configuration may pass through channels. Indeed, there is strong evidence that Co^{2+}, Zn^{2+} and Cd^{2+} ions cross the gill by the same transporter as Ca^{2+} (Comhaire et al., 1994). On the other hand, endocytosis represents one of the basic systems of nutrition in which the pollutants associated to food or sediments are transported into the cytosol and therefore is more important in filter-feeding organisms. The valence of metal ions (free metals) and their propensity to bind to organic material limits their ability to traverse cell membranes without interacting with membrane protein constituents. It should be remarked the fact that Fe, Cu, manganese (Mn) and Zn are nutrients or essential metals and cells have mechanisms to acquire them from their

extracellular environment. However, many of the transporters available for essential metals transport can be hijacked by nonessential metals such as Cd, Pb or Hg since there are no known transporters for nonessential metals (Ebany et al., 2012).

Following absorption, metals are distributed by the blood to different tissues. Five primary factors control the distribution of xenobiotics from blood to peripheral tissues: the physicochemical characteristics of the compound (e.g., pKa, lipid solubility and molecular volume), the gradient between blood and tissues concentrations, the ratio of blood flow to tissue mass, the relative affinity of the chemical for blood and tissue constituents, and the activity of specific transport proteins (Kleinow et al., 2008). To better understand these processes some of the major proteins involved in cellular uptake, retention and excretion are described below. The consequences of this net balance are the bioaccumulation and magnification. Overall, most metals are not expected to be biomagnified to any significant degree; however, lipophilic organic metals that are not easily biodegraded (e.g., triphenyltin and organoselenium) do so (Kennedy, 2011). For example, MeHg biomagnifies; it has high uptake and low elimination rates and attains its highest concentration at the top of the aquatic food chain in large predatory species such as the tuna, swordfish and sharks (Kennedy, 2011).

2.3. Biological effects and toxicodynamics

Metals are systemic toxicants and may be teratogenic, neurotoxic, cardiotoxic, and/or nephrotoxic (Kennedy, 2011). In addition, they disrupt metabolic processes by altering a number of homeostatic processes including antioxidant balance, binding to free sulfhydryl groups, and competing with cations for binding sites on enzymes, receptors and transport proteins. In the present Thesis we focus on nonessential metals, specially, Cd, Hg, Pb and As.

2.3.1. Cadmium

Cd is a nonessential metal causing great toxicity and represents the major aquatic pollutant in many parts of the world (Patrick, 2003). Cd is increasingly used in various industries such as the production of the nickel-Cd batteries and to a lesser extent in pigments, coatings, stabilizers for plastics and non ferrous alloys, and photovoltaic

devices (Waisberg et al., 2003). Cd has been categorized as a group 1 carcinogen by the International Agency for Research in Cancer (IARC) and as an eventual human carcinogen (group B1) by the Environmental Protection Agency (EPA) (Fotakis and Timbrell, 2006). The acute toxicity of Cd is highest at neutral pH, with EC_{50} (effective dose causing the 50% population death) values of 0.8–48 mg/L for freshwater fish. Marine species appear to be more tolerant, likely due to the effects of higher pH and other ions (e.g., Ca^{2+}) on Cd bioavailability. For marine species, acute EC_{50} values range between 200 and 700 mg/L in water.

In teleost fish, there is strong evidence that at least Cd traverses the gill epithelium through the chloride cells in the gills (Comhaire et al., 1994; Hogstrand et al., 1994) and uses various routes of entry (Figure 1). For example, divalent metal transport 1 (DMT1) is likely to transport free Cd (Kwong et al., 2010) whilst Cd bound to proteins can be taken up by endocytosis, especially in hepatocytes (Bressler et al., 2004). Furthermore, Cd^{2+} is a Ca^{2+} analogue and has the ability to impair calcium transport by competitive binding and selective inhibition of Ca^{2+}-ATPase transporters (Niyogi et al., 2008). In addition, zinc transporters (ZIP) have also been shown to transport Cd in mammalian cells (Himeno et al., 2009). Cd has been shown to form sulphur-conjugates with thiols, such as cysteine and glutathione (GSH), and these conjugates can act as molecular homologues, which can be transported by the amino acid or organic cation/anion transporters (Zalups and Ahmad, 2003). Following that, several molecules such as plasmatic albumin (Bentley, 1991) or metallothioneins (MTs) bind Cd in the plasma and Cd is gradually transferred into the erythrocytes, until the equilibrium is reached between the erythrocyte pool and the plasma pool of Cd. Finally, when Cd is absorbed by the body it is transported primarily to liver and kidney tissues, which remain for a number of years, while a small portion is excreted in the urine and faeces.

The main toxic effects of Cd in fish occur in gills and kidney resulting in osmoregulatory disturbances specifically hypocalcaemia (Zohouri et al., 2001). Furthermore, Cd is an endocrine modulator which produces reduced growth and effects on reproduction (Kim et al., 2004). Furthermore, Cd affects haematological parameters resulting in anaemia, and produces histological alterations and skeletal deformities. Cd is also immunomodulatory, inhibiting and stimulating different immune system components (Guardiola et al., 2013a; Voccia et al., 1996; Zelikoff et al., 1995).

2.3.2. Mercury

Hg is one of the most toxic metals. It combines with other elements (i.e. chlorine, sulphur, or oxygen) to form white powders/crystals, known as inorganic Hg salts. Elemental Hg also combines with carbon to form organic Hg and compounds. In the environment, Hg is released from natural and anthropogenic sources (Clarkson and Magos, 2006). Inorganic Hg enters the air from mining ore deposits, burning coal and waste, and from manufacturing plants. It enters the water or soil from natural deposits, disposal of wastes, and the use of Hg-containing fungicides. Hg is maintained in the upper sedimentary layers of sea and lake-beds and is methylated and thus transformed to the highly toxic species MeHg by sulphate-reducing bacteria. Historical use of Hg compounds includes fungicides, topical antiseptics, vaccine preservatives, disinfectants, laxatives, diuretics, nasal sprays, dental amalgams, batteries, thermometers, skin-lightening creams, cosmetics and other biomedical applications. Due to the information on the toxic effects of Hg its use has been reduced (Clarkson and Magos, 2006).

Regarding its bioaccumulation, concentrations of MeHg are magnified within the food chain, reaching concentrations in fish 10,000- to 100,000-fold greater than in the surrounding water (Clarkson and Magos, 2006). The primary target tissues for Hg are the central nervous system (CNS) (Clarkson et al., 2003) and the kidney, triggering loss of appetite, brain lesions, cataracts, abnormal motor coordination, and behavioural changes, alterations that lead to the fish to have impaired growth, reproduction, and development.

Hg uptake can be passive or energy-dependent, depending on the Hg species (Aschner et al., 2010) (Figure I). Concretely, in plasma, MeHg binds reversibly to cysteine amino acid and therefore to sulphur-containing molecules such as glutathione. The cysteine-bound form is of particular interest because it is transported by a L-neutral amino acid transporter system into the cells of sensitive tissues such as brain (Kerper et al., 1992). In the gastrointestinal tract, ingested MeHg is efficiently absorbed and its distribution to the blood is complete within approximately 30 h, and the blood level accounts for about 7% of the ingested dose. The brain is the primary target site for MeHg and approximately 10% is retained in the brain with the remainder transported to the liver and kidney where it is excreted through bile and urine. In rainbow trout, however, 90% of whole-blood MeHg is bound to the beta-chain of hemoglobin in red blood cells

(Giblin and Massaro, 1975). In addition, MeHg readily binds to MTs and metalloproteins with cysteine residues displacing Zn^{2+} (Aschner et al., 2010; Farina et al., 2011). The primary mechanism of MeHg as well as its specificity has yet to be identified. MeHg-cysteine conjugates have shown increased cellular efflux, presumably due to the generation and involvement of GSH.

2.3.3. Lead

Pb is a common contaminant in the natural environment that can enters the water column through geologic weathering and volcanic action, or by various anthropogenic practices including smelting, coal burning and use in gasoline, batteries and paint (ATSDR, 2007). Contamination of water through anthropogenic practices is the primary cause of Pb poisoning in fish (Monteiro et al., 2011; Rogers and Wood, 2004).

Pb uptake is modulated by dietary iron status, which alters expression of iron transport proteins such as DMT1 (Bradman et al., 2001). Once in the bloodstream, Pb is taken up by erythrocytes through anion exchange (Bannon et al., 2000) and Ca^{2+} uptake channels (Calderón-Salinas et al., 1999). Pb is actively exported from erythrocytes via Ca^{2+} ATPases (Simons, 1993). In plasma, the majority of Pb associates with albumin or sulfhydryl compounds such as cysteine, citrate, glutathione, and histidine (Al-Modhefer et al., 1991) (Figure I). Following exposure, Pb is distributed in tissues, and within weeks fish excrete nearly 99%.

Pb can be bioconcentrated from water, but it does not biomagnify and concentrations tend to decrease with increasing trophic level. Pb causes cancer, adversely affects liver and thyroid function, and decreases disease resistance (ATSDR, 2007). Other effects include muscular, spinal, and neurological degeneration (Tiffany-Castiglioni, 1993), growth inhibition, reproductive problems, and hematologic effects such as disruption in haemoglobin synthesis because Pb inhibits delta-aminolevulinic acid dehydratase (ALAD), an enzyme critical for the incorporation of iron into heme in erythrocytes (Alves and Wood, 2006).

2.3.4. Arsenic

As, a toxic metalloid, occurs naturally in the environment and is found in soil, air and water (Duker et al., 2005; Huang et al., 2004). Organic forms occurring in the environment are generally considered nontoxic, whereas inorganic forms (As^{3+} and As^{5+}) are toxic (Cervantes et al., 1994). As accumulation in the environment has increased due to human activities such as fossil fuel combustion, metal smelting and mining, semiconductor and glass industries (Nayak et al., 2007; Smedley and Kinniburgh, 2002). Furthermore, industrial sources of inorganic As compounds are insecticides, weed killers, fungicides, antifouling paints and wood preservatives (Ebany et al., 2012).

The route by which these metalloids enter the cell has been a subject of debate in the literature. Initially, it was thought that metalloids enter the cell only by passive diffusion; however, there is now a wealth of evidence indicating that special integral membrane proteins facilitate the uptake/efflux of As into/from the cell (Zangi and Filella, 2012). Indeed, uptake of As^{3+} is mediated by the aquaglyceroporins (AQPs) which are normally responsible for transporting small uncharged molecules such as glycerol, urea and water (Liu, 2010; Shinkai et al., 2009). In addition, glucose transporters (GLUT1 and GLUT2), which are glucose permeases (transmembrane proteins that facilitate the diffusion of a specific molecule in or out of the cell by passive transport), play a role in the As transport (Liu et al., 2006; Shinkai et al., 2009) (Figure I). Furthermore, the ATP-binding cassette (ABC) transporters from the ABCB (MDR1/Pgp) and ABCC (MRP1 and MRP2) subfamilies are the major pathways of As^{3+} extrusion in mammalian cells (Calatayud et al., 2012; Drobná et al., 2010; McDermott et al., 2010) but no information are available in fish cells.

Environmental As primarily accumulates in retina, liver and kidney of exposed fish. There are reports suggesting that As induces oxidative stress (Bhattacharya and Bhattacharya, 2007), liver inflammation (Pedlar et al., 2002), hypertrophy, production of stress proteins, apoptosis of fish hepatocytes (Datta et al., 2007) and alterations of the immune system (Ghosh et al., 2007; Guardiola et al., 2013b).

2.4. Toxicological mechanisms of metals

2.4.1. Cellular protection and bioaccumulation

Most biochemical defenses respond to cellular injury by increasing levels of proteins through self-regulating signal transduction mechanisms. In the case of metal toxicity the importance of MTs, heat-shock proteins (HSPs) and ATP-binding cassette (ABC) transporters are of great relevance in the binding, accumulation and excretion of metals in the cells.

MTs are a family of ubiquitous low-molecular weight cytosolic proteins that contain molecular-conserved cysteine residues with a high affinity for specific metals. These unique proteins are involved in diverse intracellular functions (Davis and Cousins, 2000), but their role in the detoxification of metals and in the maintaining of essential metal ion homeostasis, which is due to their high affinity for these metals, is mostly investigated (Klaassen et al., 1999; Templeton et al., 1991). MTs bind Zn, but with excess Cu, Cd, Ag or Hg, Zn can be easily replaced by these metals (Bebianno et al., 2007; Shaw et al., 1991). Indeed, they typically bind five to seven metal ions per molecule. Two isoforms of MT, MT-1 and MT-2, are described in all vertebrates, including fish (Smirnov, 2005; Vasák and Hasler, 2000), which are present almost in all types of soft tissues including the blood (Masters et al., 1994; Moffatt and Denizeau, 1997). MT synthesis varies with fish species, age and an analysed tissue (Navarro et al., 2009). External factors, such as season, temperature, and diet, can also effect MT induction (Rotchell et al., 2001).

MTs are inducible by some metals, stress hormones, oxidants and inflammation. The induction of MTs may not only increase the tolerance of fish to metals, but also decrease metal excretion rates due to sequestration; ecologically, this may increase the potential for bioconcentration within a fish and transfer through a trophic food chain (Kennedy, 2011). MTs can also help to damaged cells to survive by inhibition of apoptosis increasing the survival of the organism (Dutsch-Wicherek, 2008; McGee et al., 2010). Additionally, several studies support an antioxidant function for MTs, including inducibility by hydrogen peroxide and the ability to scavenge ROS (Klaassen et al., 2009). The role of the MTs as antioxidant function will be discussed later.

On the other hand, cellular stress proteins, originally known as heat shock/stress proteins (HSPs), are key components in modulating stress responses (Sathiyaa and Vijayan, 2003). They are highly conserved molecular chaperones, ubiquitously expressed, belonging to distinct multigenic families and, according to their molecular size, have been classified into several major categories: e.g., HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs. They exert several protective and homeostatic functions within the cell, for example, in fish, like in mammals, the HSP90 and HSP70 have been related to cytoprotection, cell survival and immune response (Aneja et al., 2006; Basu et al., 2001; Mayer and Bukau, 2005) exerting a protective role (Iwama et al., 1999). Several studies *in vivo* and *in vitro* have reported the induction of HSPs after a stress response in fish (Celi et al., 2012; Roberts et al., 2010). More specifically, they play an important role in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation (shape) and prevention of unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell (Horwich, 2014). Compelling evidence suggests that HSP70 may be a useful bioindicator of general cellular stress relating to proteolysis. Although the induced synthesis of the protein is transient, the turnover is much less rapid and the proteins tend to accumulate upon continued cellular stress (Sanders, 1993). In addition, the kinetics of induction appear to be longer following chemical-induced stress compared to heat-induced stress, and recovery is not achieved until several days following exposure to metal, presumably due to the accumulation of the metal into the cells (Niemi and McDonald, 2004). Overall, significant gaps remain in characterizing basal activities of fish HSPs, especially regarding potential susceptibility, gender-related, and developmental differences. Thus, it is suggested that these proteins are used in conjunction with other acute phase protein markers (i.e., MT) and enzymatic systems to transform the chemicals into a more easily excreted form (biotransformation enzymes) what is of critical importance in the detoxification pathway.

Finally, transport proteins include multidrug resistance-associated proteins (MRPs) and P-glycoprotein (Pgp) (Ayrton and Morgan, 2001; Szakács et al., 2008). Mrps and Pgp belong to the highly conserved superfamily of ABC transporters, which have been described for an increasing number of fish species, such as winter flounder (*Pseudopleuronectes americanus*) (Chan et al., 1992), Antarctic fish (*Trematomus*

bernacchii) (Zucchi et al., 2010) rainbow trout (*Oncorhynchus mykiss*) (Fischer et al., 2010; Zaja et al., 2008), zebrafish (*Danio rerio*) (Fischer et al., 2013; Long et al., 2011a, b), mullet (*Chelon labrosus*) (Diaz de Cerio et al., 2012), killifish (*Fundulus heteroclitus*) (Paetzold et al., 2009), Nile tilapia (*Oreochromis niloticus*) (Costa et al., 2012), channel catfish (*Ictalurus punctatus*) (Liu et al., 2013) and European sea bass (Ferreira et al., 2014). Both groups of transporters are plasma membrane-bound export pumps out of the cell however; their amino acid sequence identity and substrate specificity differs. Recent studies have described the regulation of these proteins after metal exposure in fish (Long et al., 2011b; Paetzold et al., 2009; Zucchi et al., 2010). Another way of eliminating cellular metals is by biotransformation. This is a two-phase process of enzymatic reactions that alter the chemistry of non-polar lipophilic chemicals to polar-water soluble metabolites, leading to the detoxification and elimination of the parent compound (Shakunthala, 2010). The organ most commonly involved in the biotransformation of foreign compounds is the liver. Biotransformation reactions of xenobiotic chemicals in fish such as PAHs, have been extensively reviewed by several authors (Schlenk et al., 2008; Van der Oost et al., 2003), however, very few information regarding biotransformation of metals is available. Few studies have demonstrated an enhanced expression of ABC transporters after metal exposure in the kidney of killifish (Paetzold et al., 2009), kidney, liver and intestine of zebrafish (Long et al., 2011a, b; Zucchi et al., 2010) or after *in vitro* or *in vivo* Cd exposure in grass carp (Tan et al., 2014).

2.4.2. ROS and oxidative stress in fish

Pollutants such as metals, pesticides or petroleum are well-known inducers of oxidative stress (Lushchak, 2011; Slaninová et al., 2009), and assessment of oxidative damage and antioxidant defences in fish can reflect metal contamination of the aquatic environment (Livingstone, 2003). Oxidative stress is an unavoidable aspect of aerobic life. Life on earth has evolved leading to aerobic organisms that cannot survive without the oxygen, since it is needed for their metabolic processes. However, when this oxygen is activated becomes toxic to these organisms. The primary basis for the toxicity of oxygen lies in its propensity to undergo electron transfers that yield ROS. Among them, we can find singlet oxygen (O_2), ozone (O_3), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and reactive nitrogen species (RNS) such as

nitric oxide (NO•) or peroxynitrite ($ONOO^-$) among others (Halliwell and Gutteridge, 2015). Excess active forms of oxygen lead to the oxidative stress, which results from the imbalance between the ROS production and the elimination by antioxidant defences in living organisms (Nishida, 2011).

The two radical species ($O_2^{\bullet-}$ and OH•) are free radicals, which means that contains one or more unpaired electrons. The existence of unpaired electrons tends to make free radicals reactive (Figure 2). Superoxide radical $O_2^{\bullet-}$ is relatively weakly reactive, while hydroxyl radical OH• is extremely reactive and is among the most potentially deleterious compounds among ROS encountered in cells. Interestingly, O_2 is a free radical; however H_2O_2 is not a radical but is also an important ROS. It has the ability to generate OH• via Haber-Weiss reaction catalysed by ion (Figure 2).

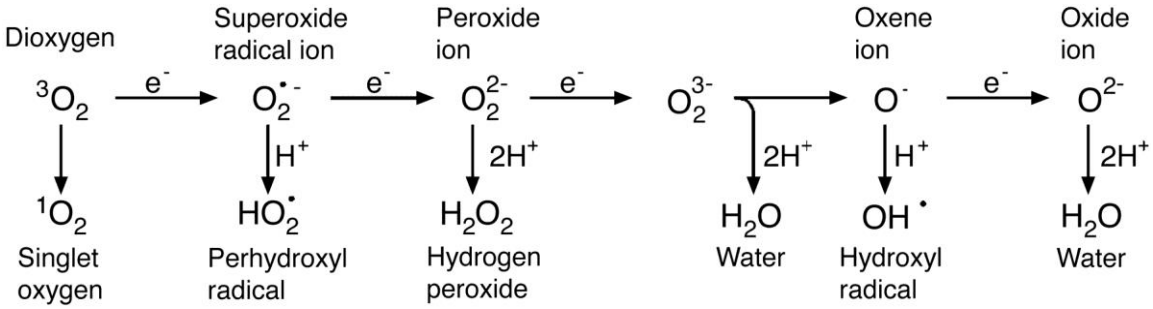

Figure 2. Generation of different ROS by energy transfer or sequential univalent reduction of ground state triplet oxygen (Apel and Hirt, 2004).

2.4.2.1. Sources of ROS

The most important sources of ROS during normal cellular metabolism are the electron transport chain of mitochondria, the endoplasmic reticulum and chloroplasts (only in plants). Thereby, in animals, mitochondrial respiration is likely the most important source of ROS. In this process, O_2 serve as the terminal electron acceptor in mitochondrial electron transport, which drives the production of high-energy adenosine triphosphate (ATP). The O_2 is reduced to H_2O, this is a four-electron reductive process that proceeds sequentially through the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical products. In this process, mitochondrial complex I and III (Figure 3) constituents are often involved (Finkel and Holbrook, 2000).

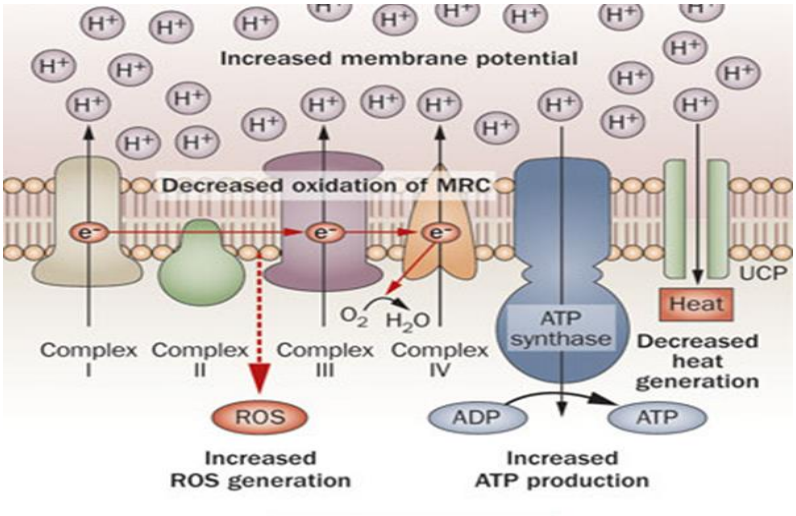

Figure 3. Mitochondrial ROS formation during the electron transport chain (Blanco et al., 2011).

Endoplasmic reticulum is another important source of ROS production. Cytochrome P450 (CYP450) plays a major role in the oxidative metabolism of endogenous compounds and xenobiotics such as PAHs (Della-Torre et al., 2012b). This multi enzymatic system catalyse oxidations by cleaving O_2 with one oxygen atom ultimately added to the substrate and the other ultimately giving rise to H_2O. In this process, two electrons provided by nicotinamide adenine dinucleotide phosphate (NADPH)-P450 reductase or cytochrome b5 are sequentially added to drive catalysis. This cycle can be uncoupled, resulting in the diversion of electrons to give rise to $O_2^{\bullet-}$ and H_2O_2 (Goeptar et al., 1995). However, to date, the effects of divalent metal ions on the activity of CYP450 enzymes have not been well understood. Cd and Pb could inhibit the CYP450 induction by different mechanism (Kim et al., 2002; Zhang et al., 2015) but further investigations are necessary to elucidate this.

Several oxidative enzymes can also generate ROS during catalysis, including 5-lypoxigenase, xanthine oxidase, nitric oxide synthase, cyclooxygenase, other NADPH dependent oxido-reductases, glycolate oxidases, D-amino oxidases, ureate oxidases, fatty acid-CoA oxidases, L-α-hydroxyacid oxidases, or lysyl oxidase (Hanschmann et al., 2013). Additionally, some molecules such as glyceraldehyde, reduced flavin mononucleotide ($FMNH_2$) or reduced flavin adenine dinucleotide ($FADH_2$) can produce ROS.

Furthermore, some specific cells can also produce ROS. For example, owing to the high oxygen and iron concentrations in the cytoplasm, erythrocytes can continuously produce ROS as a result of hemoglobin oxidation to methemoglobin (Çimen, 2008; Giulivi and Davies, 2001). In this process, oxygen will release as $O_2^{\bullet-}$ and concomitantly yield Fe (III) in the heme group. Other specific cells producing ROS are leucocytes. In the context of oxidative stress, ROS are cast as bad actors, but it is important to note that ROS play positive roles as well. In the respiratory burst process, in activated leucocytes (neutrophils and macrophages), the production of ROS is employed in the phagocytic activity of these cells against bacteria (Babior, 2000). During this process, NADPH complexes present in cell membranes, mitochondria, peroxisomes, and endoplasmic reticulum is oxidized to $NADP^+$ by NADPH oxidase enzyme. Electrons from cytoplasmic NADPH are translocated across a redox chain to reduce O_2 to superoxide anion $O_2^{\bullet-}$ inside the phagosome or extracellularly (**Figure 4**).

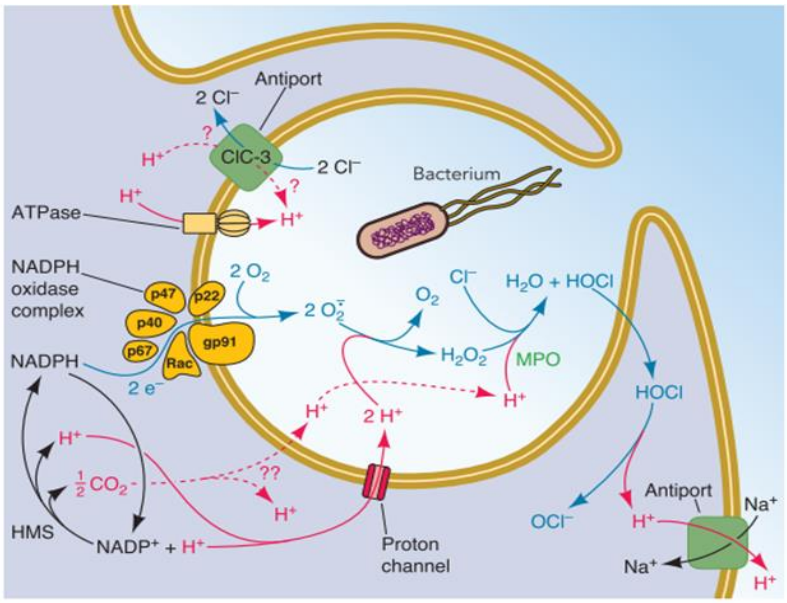

Figure 4. The main molecules and trasporters thought to participate in the respiratory burst. We can appreciate a phagocyte engulfing a bacterium into a nascent phagosome, which will proceed to close and become intracellular (DeCoursey, 2010).

The superoxide thus may subsequently be converted, spontaneously or enzymatically, into a number of different ROS such as H_2O_2, OH• or O_2. In addition, the enzyme myeloperoxidase (MPO), which occurs in neutrophils but no in macrophages (Hampton et al., 1998), in the presence of H_2O_2 catalyses the oxidation of chloride ion (Cl^-) to hypochlorous acid (HOCl), a potent bactericidal oxidant. Together, the ROS produced

during the respiratory burst are potent microbicidal agents (Babior, 2000). Moreover, release of ROS by activated phagocytic cells can be stimulated after pollutant exposure in fish (Fatima et al., 2000).

2.4.2.2. Antioxidant defences

To counteract the negative effects of ROS all aerobic organisms have evolved a diverse array of mechanisms to minimize their impacts. These mechanisms include enzyme and nonenzimatic systems that act to remove and minimize the toxic effects of ROS, whose control depends tighltly on the levels of substrates and are necessary to maintain the redox state of the organism.

Superoxide dismutase (SOD) was the first isolated enzyme able to scavenge ROS (McCord and Fridovich, 1969). This enzyme becomes $O_2^{\bullet-}$ into H_2O_2 (Figure 5) and contain three distinct forms of SOD with different cellular location: copper/zinc SOD (CuZnSOD), mainly in the cytosol but minor presence in lysosomes and the nucleus; mitochondrial manganese SOD (MnSOD); and extracellular SOD (ECSOD), which appears to play a critical role in protecting NO• from $O_2^{\bullet-}$. Numerous studies in fish have demonstrated the presence of CuZnSOD or MnSOD including the flat head grey mullet (*Mugil cephalus*) liver (Orbea et al., 1999), killifish (Meyer et al., 2003) or sea bass (Roméo et al., 2000) among others.

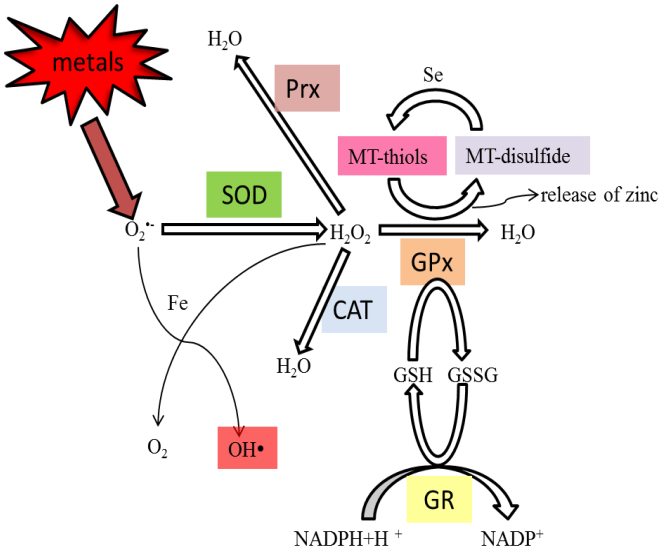

Figure 5. An overview of ROS generation and key enzymatic antioxidant defences. Superoxide dismutase (SOD), catalase (CAT), peroxiredoxins (Prx), glutathione peroxidase (GPx), glutathione reductase (GR), metallothionein MT).

Catalase (CAT) is an oxidoreductase enzyme able to reduce the hydrogen peroxide to water (Figure 5) (Aebi, 1984). It is found in the peroxisomes where prevents damage to them and also hampers the movements of H_2O_2 to other locations in the cell. The presence of CAT in fish has been also widely documented (Altikat et al., 2014; Dorval and Hontela, 2003; Orbea et al., 1999).

Glutathione peroxidase (GPx) has a broader distribution within cells. The reactions catalysed by GPx involve the reduction of a peroxide substrate to water (Figure 5), coupled with the oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG) by the glutathione transferase (Chance et al., 1979). GSH can also directly scavenge ROS, including O_2, OH• and $ONOO^-$ (Griffith et al., 1978; Halliwell and Gutteridge, 2015), with the thiol (–SH) group provided by cysteine as the active moiety that undergoes oxidation. Furthermore, glutathione reductase (GR) enzyme catalyses the reduction of GSSG to GSH. In the case of GPx, it has been characterized in liver of Japanese sea bass (*Lateolabrax japonicas*) (Nagai et al., 2002), gills, and posterior kidney of channel catfish (Gallagher and Di Giulio, 1992) or gilthead seabream larvae (Sassi et al., 2013) among others.

Peroxiredoxins (PRXs or PRDXs), first described in yeast (Kim et al., 1988), are a family of antioxidant enzymes that protect cells from oxidative damage by reducing H_2O_2, peroxynitrite, and lipid peroxidation, as well as by scavenging thiyl radicals (Figure 5). After CAT, they are probably the most important hydrogen peroxide-scavenging enzymes in cells. Depending on their structure they show differential compartmentalization (Valero et al., 2015a). The presence of PRXs has been found in rainbow trout (Mourich et al., 1995), bluefin tuna (*Thunnus thynnus*) (Sutton et al., 2010), catfish (Li and Waldbieser, 2006), gilthead seabream (Pérez-Sánchez et al., 2011), European sea bass (Esteban et al., 2013) or Atlantic salmon (Loo et al., 2012).

On the other hand, the hypothesis that MT functions as antioxidant against ROS and RNS has received extensive experimental support from many *in vitro* studies (Klaassen et al., 1999; Ruttkay-Nedecky et al., 2013). Studies using a cell-free system have demonstrated the ability of MT as free radical scavengers (Cai et al., 2000; Thornalley and Vasak, 1985). The cluster structure of Zn-MT provides a chemical basis by which the cysteine ligand can induce oxido-reductive properties (Kang, 2006). Under physiological conditions, Zn bound to MT is released through oxidation of the thiolate

cluster when the environment becomes oxidized. Formation of MT-disulphide would be subjected to degradation; however, when the oxidized environment became reduced (for example, an increase in the GSH/GSSG ratio), MT disulphide is reduced to MT-thiol (Figure 5). This reduction route is greatly enhanced in the presence of Se while the presence of Zn quickly reconstitutes MT. This process constitutes the MT redox cycle, which plays a crucial role in the biological function of MT (Kang, 2006; Schwarz et al., 1995).

Furthermore, other important nonenzymatic antioxidants such as ascorbic acid (vitamin C), tocopherols (vitamin E components) and carotenoids (vitamin A, or retinol, precursors) have been less studied in relation to fish toxicology but likely they play an important role (Bell et al., 2000; Olsen et al., 1999; Parihar and Dubey, 1995; Payne et al., 1998; Tocher et al., 2002).

2.4.2.3. Biomarkers of oxidative stress

When aquatic ecosystems are polluted, fish will be inevitably affected (Amado et al., 2006; Carvalho-Neta et al., 2015). In fish, the effects of pollutants can be assessed using different biomarkers. These markers are defined as biological responses to the effects of pollutants that can be used to identify the early signs of damage to organisms (Carvalho-Neta et al., 2015). Biomarkers can be measured at the molecular, cellular, or even organism level, and may be specific to certain pollutants (Carvalho-Neta et al., 2012; Livingstone, 1993). In the present Thesis, we have focused on cellular protection and antioxidant gene expression changes as biomarkers of the oxidative stress. Indeed, it is known that expression of many genes such as cellular proto-oncogenes, GR, heme oxygenase, MnSOD and ECSOD, MTs or GPx among others are up or down-regulated at the level of mRNA transcription under cellular oxidative stress (Brady et al., 1997; Schull et al., 1991). Specially, we have focused on the alterations at gene level of mRNA encoding for proteins such as MTs, Hsps, SOD, CAT, GR or Prxs among others after exposure to metal. We have chosen them because of the correlation they have with levels of exposure to Cd, MeHg, Pb or As (Hsu et al., 2013; Rajeshkumar et al., 2013; Selvaraj et al., 2013).

2.4.2.4. Deleterious cellular effects of ROS

One of the most common consequences of ROS overproduction is the peroxidation of lipids and proteins. First, lipid peroxidation is perhaps the most studied consequence of ROS imbalance (Devyatkin et al., 2006; Kim et al., 2014). Of particular interest are the effects on polyunsaturated fatty acids (PUFAs) as part of the membrane phospholipids, and therefore important in all the membranes including the cell and organelles such as mitochondria, lysosomes and endoplasmic reticulum. Firstly, ROS combines with a hydrogen atom from a lipid to make a fatty acid radical that rapidly react with O_2 yielding a lipid peroxyl radical (ROO·), which can react with another PUFA, thereby abstracting hydrogen, becoming lipid peroxide (LOOH), and generating another second lipid radical. This second radical can also react with O_2 to yield ROO·, and this process can be repeated many times, constituting a free-radical chain reaction termed propagation of lipid peroxidation (Di Giulio and Meyer, 2008). Consequences of membrane lipid peroxidation include decreased membrane fluidity, increased permeability resulting in inappropriate leakage of some molecules, and inhibition of membrane-bound enzymes (Niki et al., 2005). In a similar way, protein peroxidation occurs and leads to enzyme inactivation, alteration of receptors and other proteins involved in signal transduction, and perturbed ion homeostasis (Dalle-Donne et al., 2003).

Additionally, ROS can interact with DNA and produce another form of DNA damage, oxidative DNA damage. The consequences of oxidative DNA damage vary considerably with the identity of the base oxidized, the base sequence surrounding the modified base, and the efficiency of DNA repair systems available (Begley and Samson, 2004). Oxidative DNA damage can lead to mutations and cancer initiation (Marnett, 2000).

Finally, in the cases in which the oxidative stress situation, if the prooxidant-antioxidant balance is in favour of the former, is maintained o exacerbated the cell death occurs.

2.4.3. Cell death: apoptosis and necrosis

Cell death constitutes one of the key events in biology. At least two modes of cell death can be distinguished: apoptosis and necrosis (Kroemer et al., 1998).

Apoptosis is considered as an ongoing normal event in the control of cell populations. Essentially, it occurs when cellular damage, including damage to genetic material, has exceeded the capacity for repair (Rana, 2008). By contrast, necrosis is always the outcome of severe and acute injury such as infection, toxins, or trauma which results in the unregulated digestion of cell components (autolysis). Morphological differences between apoptosis and necrosis cell death are shown in Figure 6.

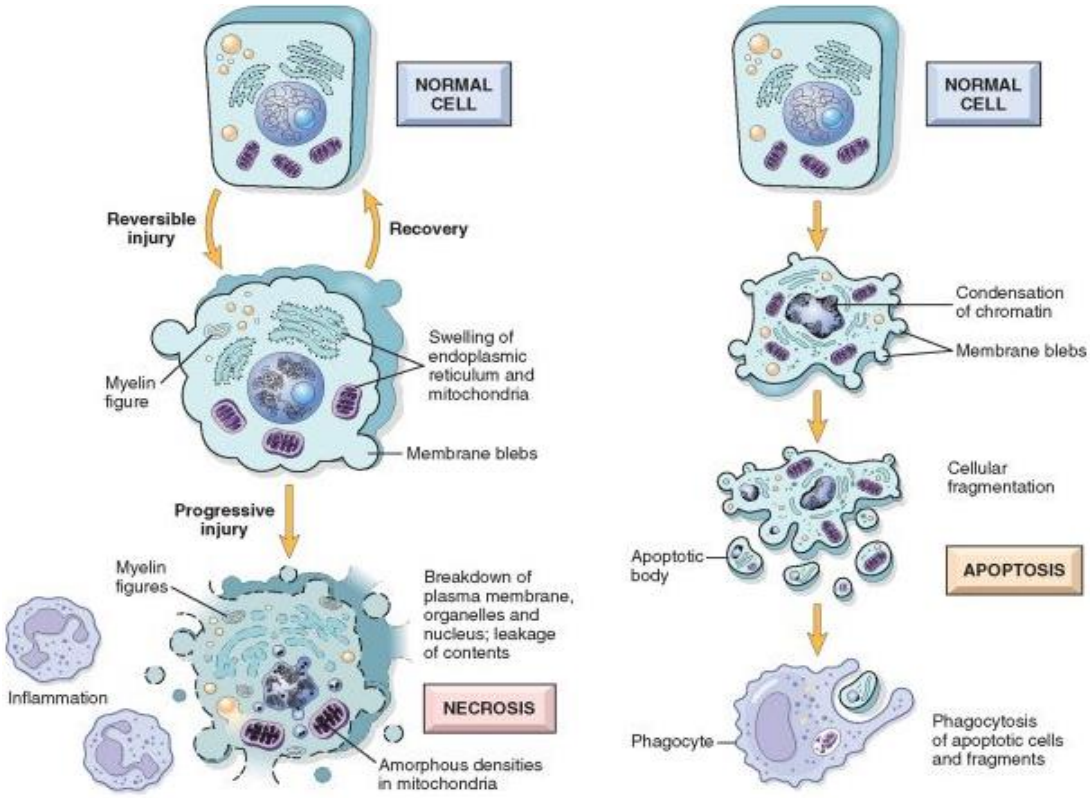

Figure 6. Morphological differences between apoptosis and necrosis cell death (Kumar et al., 2009).

Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Apoptosis is mainly mediated through one of two signalling cascades termed the

intrinsic and the extrinsic pathway (Figure 7). During the extrinsic pathway, external signals or ligands interact with tumor necrosis factor receptor (TNFR), Fas, death receptors, and decoy-receptors at the plasmatic membranes leading to the recruitment of Fas-associated death domain protein (FADD) and pro-caspase 8, followed by dimerization and activation of caspase 8, which then directly cleaves and activates executor caspases 3 and 7 (Elmore, 2007). Alternatively, the intrinsic pathway is activated by stimuli such metals or ROS, that lead to outer mitochondrial membrane permeabilization, the release of cytochrome C which binds to apoptotic activating factor-1 (Apaf-1) and activates caspase 9 leading to the activation of caspase 3 (Lu et al., 2013). In addition, mitochondrial membrane permeability is regulated through a family of anti-apoptotic (Bcl-2, Bcl-xL, etc) and pro-apoptotic (Bad, Bax, Bak, Bid, etc) proto-oncogenes (Borner, 2003). Once activated, Bax is inserted into the mitochondrial membrane and increase membrane permeability, thereby promoting apoptosis (Tait and Green, 2010). The anti-apoptotic protein Bcl-2 inhibits the ability of Bax to increase membrane potential (Youle and Strasser, 2008). As such, the balance of these protein regulators may determine cell fate.

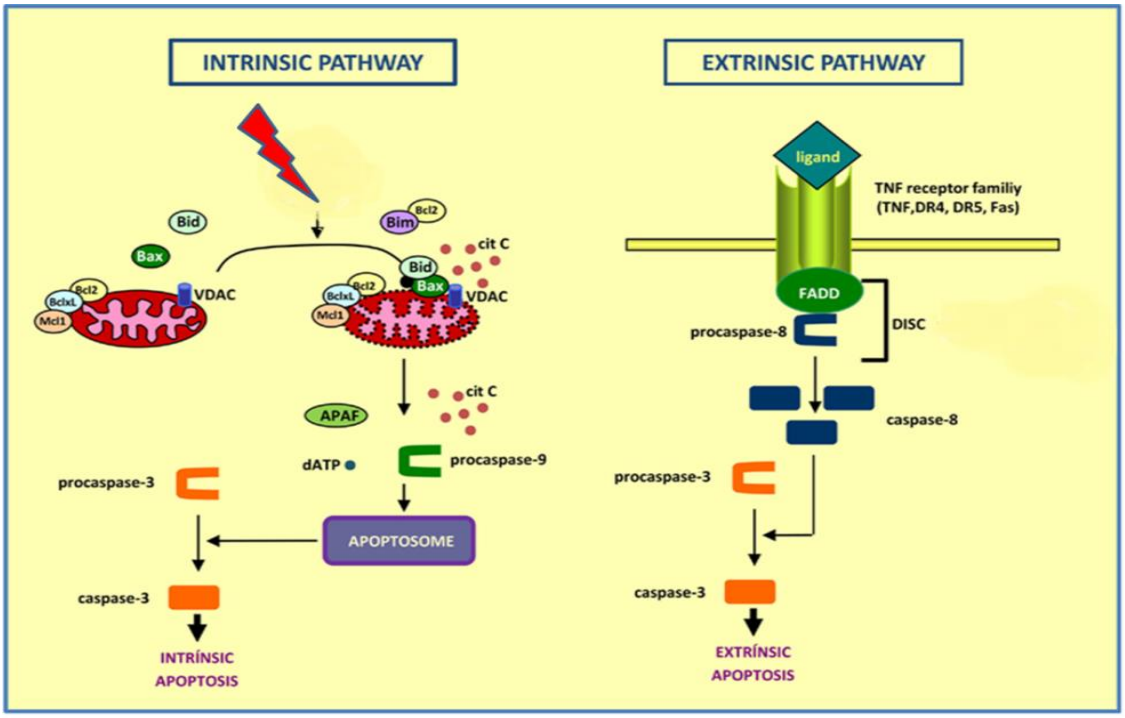

Figure 7. Diagram showing the intrinsic (left) and extrinsic (right) apoptotic pathways. Modified from Gómez-Sintes et al., (2011).

Necrosis fails to display a stereotyped morphology (except for the early rupture of plasma membranes) and has historically been regarded as an unregulated cell death that is induced by nonspecific and nonphysiological stress (Hitomi et al., 2008). Necrosis has been typified by ATP depletion and plasma membrane permeabilization (Figure 7) (López et al., 2003; Yang et al., 2007).

Although apoptosis and necrosis are the most usual cell death mechanisms there are others but we will focus only on those induced or related to metal toxicity. Hence, in some instances, a less clearly defined mode of cell death was described involving both apoptotic and necrotic features (Lee et al., 2006a; Sancho et al., 2006; Shih et al., 2003). Recently, a mode of programmed necrosis, termed necroptosis, has been identified, in which cell death occurs with largely necrosis-like morphological alterations but following apparently well-defined molecular signalling pathways that recruit components of the extrinsic as well as intrinsic apoptotic cell death machinery (Christofferson and Yuan, 2010; Galluzzi and Kroemer, 2008). A crucial component of this pathway is the serine/threonine kinase receptor-interacting protein-1 (RIP-1), the pharmacological inhibition of which by the small molecule inhibitor necrostatin-1 (Nec-1) was in fact key to identifying necroptosis (Degterev et al., 2005). The pharmacological or genetic inhibition of several key enzymes has been shown to deeply affect the execution of programmed necrosis. These include receptor-interacting serine/threonine-protein kinase 1 (RIP1), cyclophilin D, poly (ADP-ribose) polymerase 1 (PARP-1), and apoptosis-inducing factor (AIF). Furthermore, the Ca^{2+} entry and the ROS production trigger the PTPC (permeability transition pore complex) dependent mitochondrial permeability transition and finally, necrosis programmed cell death (Degterev et al., 2005; Galluzi and Kroemer, 2008).

In addition, some studies have also suggested the term of eryptosis to describe the suicidal death of erythrocytes (Lang et al., 2005). The eryptosis is important to avoid the hemolysis, as well as the inflammatory or immunological responses in erythrocytes destruction. Eryptosis is triggered by several stressors such a metals (Hg, Pb, Cd, As) (Kempe et al., 2005; Mahmud et al., 2009; Sopjani et al., 2008), osmotic shock (Lang et al., 2003), energy depletion (Klarl et al., 2006) oxidative stress (Lang et al., 2014) or increase of temperature (Föller et al., 2010). As noted above, metals can induce the overproduction of ROS. During the oxidative stress, energy depletion impairs the

replenishment of GSH and thus weakens the antioxidative defense of the erythrocytes (Bilmen et al., 2001). Accordingly, energy depletion similarly activates the cation channels and Ca^{2+} entry into the cell. The increase of intracellular calcium provokes the cell shrinkage, the phosphatidylserine exposure (Dekkers et al., 2002) and the activation of the calpain protein, a key indicator of eryptosis, which degrades cytoskeleton proteins (Lang and Lang, 2015) inducing the appearance of blebs, which are characteristics of apoptosis process.

2.5. Metals and molecular mechanisms of toxicity

Fish exposure to metals can activate several processes that finally lead to the cell death of the cells. Thus, the prospect of developing mechanism-based, noninvasive biomarkers is one of the goals of toxicology. These markers may provide novel clues regarding the pathophysiology of disease in fish and some potential value for ecological risk assessment. For example, genotoxicity (chromosomal aberrations or micronuclei), oxidative stress and some proteins involved in bioaccumulation or detoxification (MT in the case of metals) are key biomarkers in this field. Unfortunately, few studies have focused on the cell death biomarkers. In this Thesis, we focus on some of the most important markers of apoptosis cell death (*bax*, *bcl2*, *caspases*) at RNA transcription to better understand the toxicity mechanisms after metal exposure.

2.5.1. Cadmium

The potential role of ROS and free radicals has been highlighted as mediators for apoptotic cell death, suggesting the involvement of oxidative stress in Cd-induced apoptosis (Risso-De Faverney et al., 2004). Cd enters the electron transport chain in mitochondria, leading to accumulation of unstable semiubiquinones, which donate electrons and create superoxide radicals (Wang et al., 2004a). Furthermore, Cd also affects antioxidant enzymes, especially SOD and CAT activities (Firat and Kargin, 2010). Reduced CAT activity following Cd exposure has been reported in the kidney of European sea bass (Roméo et al., 2000), what is suggested to occurs by the direct binding of Cd to CAT. Cd can also alter the GSH oxidation in fish (Cao et al., 2010; Jia et al., 2011) affecting to its turn-over. In addition, the synthesis of MTs following Cd exposure has been described *in vivo* studies in greater amberjack (*Seriola dumerili*)

brain (Jebali et al., 2006) or liver, gill and kidney of gilthead seabream (Ghedira et al., 2010) as well as *in vitro* studies (Monteiro et al., 2011; Roméo et al., 2000).

Regarding cell death, Cd also may directly lead to the dysfunction of the mitochondria, release of cytochrome C (Li et al., 2003), activate caspase 3 (López et al., 2003) and inhibit the expression of *bcl2* and *p53* genes (Cai et al., 2004). Studies *in vivo* or *in vitro* in fish have revealed that Cd induce ROS and causes apoptosis in rainbow trout hepatocytes (Risso-De Faverney et al., 2001, 2004), zebrafish embryos (Hsu et al., 2013),common carp (*Cyprinus carpio*) skin and blood (Ferencz and Hermesz, 2015; Witeska et al., 2011), European sea bass blood leucocytes (Bennani et al., 1996; Roméo et al., 2000; Vazzana et al., 2014) or gilthead seabream leucocytes (Guardiola et al., 2013a). However, some authors have demonstrated that depending on the cell type and concentration, Cd it may induce cell death via apoptosis or necrosis. In some instances, a less clearly defined mode of cell death was described involving both apoptotic and necrotic features in fish cells (Krumschnabel et al., 2010; Risso-De Faverney et al., 2001).

2.5.2. Mercury

Several studies have shown an induction of ROS after Hg exposure *in vivo* (Mieiro et al., 2010; Monteiro et al., 2011) or *in vitro* (Sarmento et al., 2004; Voccia et al., 1994). Indeed, Hg reacts with the thiol groups of GSH, which can induce GSH depletion and oxidative stress (Elia et al., 2003; Rana et al., 1995). Thus, some studies have found alterations in the antioxidant system caused by Hg exposure including GR and GPx activities in zebra seabream (*Diplodus cervinus*) (Branco et al., 2012) or modifications in SOD, CAT, GST, and GPx activities in trahira (*Hoplias malabaricus*) (Mela et al., 2014). Very recently, MeHg exposure increased the ROS levels and decreased the antioxidant potential of gilthead seabream serum while increased the SOD, CAT and GR activities in the liver (Guardiola et al., 2016). In addition, significant alterations in the expression of the antioxidant enzyme genes *sod*, *cat*, *gst*, *gpx*, and *gr* have been observed in the freshwater fish Yamú (*Brycon amazonicus*) after Hg exposure leading to oxidation of lipids and proteins (Monteiro et al., 2010). MTs also play a protective role in response to Hg exposure. The mRNA expression of two *mt* genes was noted in the liver of common carp from a mercury-contaminated river (Navarro et al., 2009).

However, no significant correlations between total Hg content and MT levels were described by Mieiro et al. (2011) in different fish tissues from Hg contaminated area.

Furthermore, in mammals, Hg acts as a genotoxin significantly altering the expression of *prx2* gene and enhancing the sensitivity of kidney cells to apoptosis (Woods et al., 2002). Hg induces apoptosis by inhibiting mitochondrial function (Carranza et al., 2005) and releasing cytochrome C from the mitochondria to the cytosol (Lee et al., 2006b). Moreover, p38 mitogen-activated protein kinase is activated by mercury resulting in apoptosis (Kim and Sharma, 2003). However, Hg induced a mix of necrosis and apoptosis cell death in murine macrophages (Kim and Sharma, 2003). Preliminary histopathological data found tissue disorganization and necrotic catfish (*Trichomycterus brasiliensis*) cells exposed to inorganic Hg (Oliveira-Ribeiro et al., 1996) and after MeHg exposure in arctic charr (*Salvelinus alpinus*) (Oliveira-Ribeiro et al., 2002). Unfortunately, studies on the mechanisms of Hg in fish cells have not been performed even considering it is one of the most threatening metals for humans and fish are the main source of Hg.

2.5.3. Lead

Pb can induce oxidative damage through direct effects on the cell membrane, by interacting with haemoglobin, which increases the auto-oxidation of haemoglobin and auto-oxidized ALAD, interactions with GR, or through the formation of complexes with Se, which decreases GPx activity (Ercal et al., 2001). Pb can also induce the synthesis of MTs in the toadfish (*Halobatrachus didactylus*) (Campana et al., 2003) or in mud-sucker (*Prochilodus lineatus*) liver (Monteiro et al., 2011), although to a lesser degree than some other metals. Pb can also elevate the SOD activity in the crucian carp (*Carassius carassius*) (Ding et al., 2014).

In Pb-induced apoptosis in mammals, mitochondria play a crucial role. Since Pb mimics calcium, calcium overload may triggers apoptosis. Ca^{2+} and Pb both depolarize rod cell mitochondria resulting in the cytochrome C release, caspase activation and apoptosis (He et al., 2000). In rat cerebral cortex, hippocampus and cerebellum, Pb-induced apoptosis results in an increased Bax/Bcl-2 ratio (Sharifi et al., 2002). However, there is only limited evidence of direct genotoxic or DNA-damaging effects for Pb. Few studies

have provened that Pb can induces DNA damage and necrotic cell death in fish (Ferraro et al., 2004; Monteiro et al., 2011).

2.5.4. Arsenic

There exists a general consensus that As causes oxidative stress. In this sense, GSH plays a key role in the cell redox status induced by As because it is an electron donor in the reduction of As^{5+} to As^{3+}. As cell metabolism generates ROS, although the mechanisms are not clear. RNS are also involved in oxidative damage associated with As (Allen et al., 2004; Bhattacharya and Bhattacharya, 2007). Oxidative stress-induced apoptosis was suggested by Seok et al. (2007) as a possible mechanism of As toxicity in a zebrafish liver cell line (ZFL). In mouse embryonic fibroblasts, As stimulates release of cytochrome C from isolated mitochondria via Bax/Bak-dependent release (Bustamante et al., 2005). As exposure can leads to DNA strand breaks and DNA oxidation (Matsui et al., 1999; Yih and Lee, 2000). Furthermore, the nuclear factor NFkB is modulated in various cells exposed to As (Chen et al., 2001). On the other hand, studies carried out in the topminnow (*Poeciliopsis lucida*) liver cell line (PLHC-1) have showed that As_2O_3 induces apoptosis and necrosis mediated cell death through mitochondrial membrane potential damage (Selvaraj et al., 2012).

3. *In vitro* models in toxicology

Studies in goldfish (*Carassius auratus*) and minnows (*Pimephales promelas*) were the first fish species used in aquatic toxicity tests to determine the effects of chemicals *in vivo* (Penny and Adams, 1863). However, it was not until after World War II that efforts were directed towards standardizing techniques for fish acute toxicity tests (Hart et al., 1945) and established them as the workhorse for studying and monitoring the effects of pollution (Buikema et al., 1982). Nowadays, *in vivo* fish studies are employed in toxicology tests (acute, subchronic and chronic, depending on the time exposure) of many pollutants such as PAHs, PCBs, DDTs and metals among others (Guardiola et al., 2016; Monteiro et al., 2011; Velma et al., 2010). In addition, fish are also used to assess the ecological risk from these pollutants by measuring bioaccumulation (Van der Oost

et al., 2003), genotoxicity (Costa et al., 2008; Della-Torre et al., 2012a), cell death (Julliard et al., 1996); oxidative stress (Monteiro et al., 2013) or detoxification (Ferreira et al., 2014) biomarkers as well as the interplay with environmental factors (e.g., changes in temperature, dissolved oxygen) (Sassi et al., 2013). Most used fish models in toxicology include zebrafish, medaka (*Oryzias latipes*), rainbow trout, fathead minnow and Atlantic killifish (Carvan et al., 2007).

Unfortunately, the number of animals used in research has increased with the evolution of research and development in medical technology. Every year, millions of experimental animals are used all over the world but the use of various alternatives to animal testing have been proposed to overcome the drawbacks associated with animal experiments and avoid the unethical procedures. A strategy of 3Rs, (reduction, refinement and replacement) is being applied for laboratory use of animals (Doke and Dhawale, 2015). Some of the alternative methods include the use of computer models (such as computer aided drug design (CADD), structure activity relationship (SARs) or the development of cell and tissue cultures. Thus, we carried out in this Thesis the toxicological studies using fish cell lines and primary cultures *in vitro* instead of living fish.

3.1. Fish cell lines

Culture of established fish cells *in vitro* is relatively rapid, cost-effective, readily reproducible, and can be easily adapted to automated high-throughput screening technologies (Bols et al., 2005; Fent, 2001). Unlike mammalian cell lines, fish cell lines have a wider temperature range for incubation and lower metabolic rates and therefore their maintenance and manipulation requires lower care and conditions (Lakra et al., 2011). Currently, there are around 300 fish cell lines, and about half of them are commercially available. Cell lines are derived from marine (i.e. SAF-1, SPH, GF-1, CRF-1, etc.) or freshwater fish species (i.e. BF-2, WSF, GFM, and, more recently, zebrafish-derived cell lines). Specially, great interest due to their use in aquaculture settings have received fish cell lines derived from salmonid (i.e. RTG-2, CHSE-124) or cyprinid (i.e. EPC, MG-3, RG-1) fish species (Lakra et al., 2011; Segner, 2004). Cell lines are originated from different tissues (i.e. skin, gills, heart, liver, kidney, spleen, swim, bladder, brain) and are usually described either as fibroblast- or epithelial-like, based simply on cellular morphology. These cells are immortalized by different

methods: spontaneous transformation (Bodnar et al., 1998), the use of viral oncogenes (Takakura et al., 1999) or induction of the expression of telomerase reverse transcriptase protein (TERT) (Takakura et al., 1999). Fish cell lines have been mainly used in areas such as virology (Wolf, 1988) and toxicology and ecotoxicology (Schirmer, 2006; Segner, 1998) followed by their application to fish immunology (Bols et al., 2001) or biotechnology and aquaculture (Bols and Lee, 1991) among others.

Due to the advantages of using cell lines, several cytotoxic studies have been performed in response to a chemical exposure. The first use of fish cells *in vitro* to determine the cytotoxicity of chemicals was by Rachlin and Perlmutter (1968), who studied the toxic action of zinc on the FHM cell line from the fathead minnow. Since then, a number of *in vitro* cytotoxicity assays using fish cells have been developed. For example, exposure to metals has been studied in fish cell lines such as barramundi (*Lates calcarifer*) BB, fathead minnow EPC, rainbow trout RTG-2 or gilthead seabream SAF-1 (Minghetti et al., 2011; Mori and Wakabayashi, 2000); to PAHs in barramundi BB, bluegill fry (*Lepomis macrochirus*) BF-2 or rainbow trout RTgill-W1 cell lines (Brinkmann et al., 2014; Schirmer et al., 2000); to pesticides in Japanese flounder (*Paralichthys olivaceus*) FG-9307 or fathead minnow FHM cell lines (Crago et al., 2015; Li and Zhang, 2002) among others. *In vitro* cytotoxicity assessments can be readily employed to examine multiple parameters, including measurements of viability, morphology, metabolism, cell attachment/detachment, cell membrane permeability, proliferation and growth kinetics (Maracine and Segner, 1998; Schirmer et al., 1998). For example, colorimetric neutral red (NR), 3-(4,5-dimethylthizol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), propidium iodide (PI) uptake and lactate dehydrogenase (LDH) release or crystal violet (CV) methods are some examples used to evaluate lysosomal damage, mitochondrial impairment, membrane integrity and cell attachment/detachment, respectively. Other tests based on the cell attachment or changes in the cytoskeleton of fish cells are less frequent (Reinhart and Lee, 2002; Zahn et al., 1996). Apart from endpoint cytotoxicity studies, other parameters evaluated in fish cell are the cell growth (Bechtel and Lee, 1994), the genotoxicity, including chromosome aberrations, DNA damage and induction of micronuclei (Bagdonas and Vosyliere, 2006; Brinkmann et al., 2014) and xenobiotic metabolism (Segner et al., 1994). Thus, the use of multiple tests can potentially reveal mechanisms behind cytotoxicity. Interestingly, many cytotoxicity studies have positively related the *in vitro* and *in vivo* EC_{50} (effective concentration

producing 50% cell death) values in fish exposed to different contaminants demonstrating the goodness of the fish cell lines in this field (Castaño et al., 1996; 2003; Clemedson and Ekwall, 1999; Fent, 2001; Segner, 2004; Vega et al., 1996). Thus, the use of fish cell lines in the field of toxicology not only will help to extrapolate the potential effects on fish biology but also dilucidate the mechanisms involved in the toxicity.

3.2. Primary fish cell cultures

Primary cultures are initiated directly from the cells, tissues or organs of fish, therefore, cultures of cells may represent a more appropriate and realistic models of tissues *in vivo* (Freshney, 2011). Cultures can be maintained as primary cell cultures from few days to several months or even for few years. Unlike mammalian primary cell cultures, few piscine primary cultures have been employed in aquatic toxicology. Furthermore, the majority of the *in vitro* studies using primary cultures have been performed with freshwater species, and only a few studies used primary cell cultures from marine species. Despite of, some examples of marine primary cell cultures include the use of hepatocytes (Naicker et al., 2007; Smeets et al., 2002; Winzer et al., 2000) and those derived from blood, muscle, brain, skin or gills (Bols et al., 1994; Minghetti et al., 2014; Rakers et al., 2011). Interestingly, freshly isolated leucocytes have been also used for immunotoxicological studies due to the scarcity of fish immune-derived cell lines, which will be discussed later. Another very promising primary cell culture is performed with fish erythrocytes since they represent cells in contact with pollutants when they enter the body and are dispersed through the body. Toxicological effects of many toxics have been widely described in human erythrocytes (Hunaiti et al., 1995; Kempe et al., 2005; Sopjani et al., 2008), but less in fishes. Owing to their sensitivity to chemicals and the fact that fish erythrocytes are nucleated (Kondera and Witeska, 2013) possess mitochondria (Moyes et al., 2002), exhibit good resistance in primary culture and are easy to handle, fish blood cells constitute a very interesting cellular model for toxicological studies *in vitro.* Additionally, experimental fish are not sacrificed which turns them into a valuable alternative to fish bioassays and contributes to the three R's. *In vivo* toxicological studies in fish erythrocytes have reported the effects of various chemicals in the morphology, hemolysis, nuclear deformation, amitosis (Bogé and Roche, 1996; Kondera and Witeska, 2013) and genotoxic damage (Bagdonas and

Vosyliere 2006; Monteiro et al., 2011). However, fewer studies have evaluated the toxicological role of metals on fish erythrocytes *in vitro*, which could provide basic information on the nature of the tested agents and/or the cellular response.

4. Immunotoxicology

The increased knowledge of fish immunology and the potential effects that pollutants may exert in their immune response have developed the specific field of toxicology called immunotoxicology (Carlson and Zelikoff, 2002). Most immunological research involving fish species has been concerned with either their phylogenetic or economic importance; thus, such groups as agnathans, elasmobranchs, and chondrosteans have been studied in attempts to elucidate the evolution of the vertebrate immune system. Also, species such as salmonids, carp, catfish, seabream and sea bass have received interest mainly due to their use in aquaculture industries and human consume. Though fish immunotoxicology is a relatively new field of study, is rapidly expanding as more and more techniques and reagents become available for use also in teleost species (Zelikoff et al., 2000).

4.1. Fish immunology

The immune system is composed of cells and molecules that are responsible for the immune reaction against foreign agents, including microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and macromolecules (proteins and polysaccharides). In general, the immune system of fish is very similar to higher vertebrates, but has some important differences. In all vertebrates, including fish, the immune response may be of two types: innate (also known as natural or nonspecific) and adaptive (also called acquired or specific), whereas invertebrates possess only innate immune response (Tort et al., 2003). The fish constitute the first group of vertebrates displaying cellular and humoral responses that have the characteristics of specificity and memory (Uribe et al., 2011). Regarding to the innate immune response, this includes physical barriers (epithelium and mucous membranes), cellular effectors (phagocytic cells and nonspecific cytotoxic cells) and humoral factors (complement and other acute-phase proteins), acting as first line of defense against infection until the

specific response is activated, whilst the adaptive response has cellular (lymphocytes) and humoral (secreted antibodies) components which appears exclusively in vertebrates (Figure 8) (Tort et al., 2003). Both responses are greatly interconnected and involve a wide variety of innate and adaptive components.

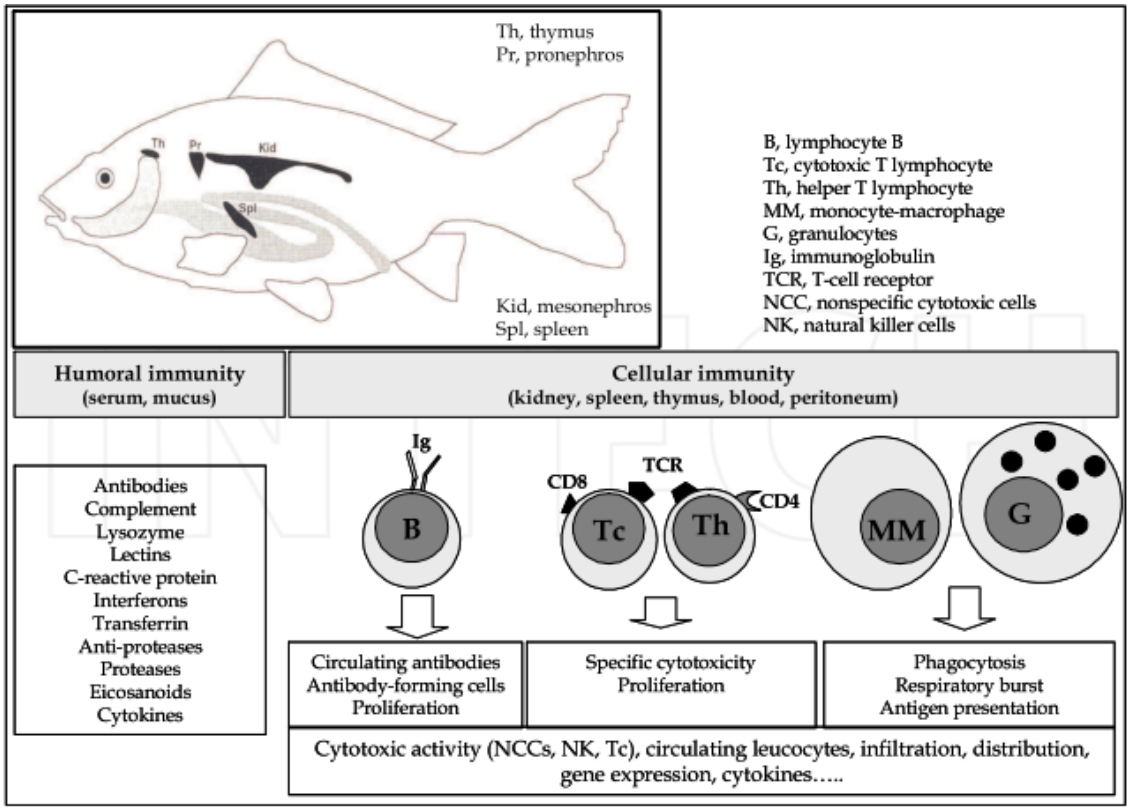

Figure 8. Fish immune system organization and representative humoral and cellular immune responses used in immunotoxicological studies (Cuesta et al., 2011).

The organs of the immune system of fish are also essentially similar to those of mammals, comprising primary (where lymphocyte maturation occurs) and secondary organs (where, mainly, mature lymphocytes come in contact with antigens) (Alberts et al., 2002). The kidney, a primary organ, is the main hematopoietic organ, which is structured in three parts: the anterior or cephalic (head-kidney, HK), with lymphoid and hematopoietic function; posterior or caudal, with renal function; and the intermediate, which shares both functions. The second most important primary organ is the thymus, which appears near the gills and is mainly composed of T lymphocytes, being considered as the main source of mature T lymphocytes. Moreover, the spleen is the

main secondary organ of teleost fish. It shows few lymphocytes that greatly increase after antigen administration, finding both T and B lymphocytes (Alberts et al., 2002). An important component of the lymphoid tissue is associated with mucus, forming the MALT (mucosa-associated lymphoid tissue) that is dispersed in the skin, gut and gill (Salinas et al., 2011).

The humoral immunity is played by proteins and glycoproteins with defence functions which are found in serum, mucus and eggs (Salinas et al., 2011). Among the humoral factors of the innate immune system the most important is the complement system that consists of a complex cascade of enzymatic glycoproteins, which acts signaling the presence of potential pathogens and contributing to their degradation through chemotaxis and opsonization. The complement system is well developed in fish and includes classical, alternative and lectin pathways. Other important humoral factors are lytic enzymes or lysins including hydrolases, such as lysozyme and chitinase, cathepsin and bacteriolytic enzymes (Saurabh and Sahoo, 2008), lectins, agglutinin, pentraxins, acute-phase proteins (C-reactive and serum amyloid P protein), transferrin, antiproteases or ceruloplasmin. It is important to mention the presence of many antimicrobial peptides (AMPs), which are low-molecular-weight peptides that have bactericidal properties against different pathogens (Fernandes and Smith, 2004; Maier et al., 2008). Regarding the acquired humoral immunity are immunoglobulins (Ig antibody) expressed as membrane molecules of B lymphocytes or secreted into the plasma. Until recently, it was believed that the fish had only two classes of immunoglobulins, IgM and IgD. Nevertheless, after the analysis of multiple genomes of teleost fish, it was discovered a new immunoglobulin isotype called IgT in rainbow trout (Hansen et al., 2005), IgZ in zebrafish (Danilova et al., 2005) and IgH in Japanese pufferfish (*Fugu rubripes*) (Savan et al., 2005). Recently, the protein structure, production and potential role in immunity of IgT was studied (Xu et al., 2013, 2016) showing that the IgT of rainbow trout is an immunoglobulin specialized in the immune responses of the intestinal and skin mucosa, while IgM appears to be specialized in systemic immunity. The antibodies are responsible for antigen neutralization, precipitation and agglutination, opsonization and activation of the classical complement pathway. A crucial aspect of the immune response is specific immunological memory, thus the secondary antibody production in teleosts is often more extensive and rapid

than the primary, but this immunological memory is not as developed as in mammals (Uribe et al., 2011).

The immune system cells are classified into three main types: monocyte-macrophages, granulocytes and lymphocytes. These cells interact between them resulting in different innate and adaptive immune responses (Cuesta et al., 2011). Cell-types involved in nonspecific immune response of fish are monocyte-macrophages, granulocytes, platelets and cytotoxic (NCC or NK) cells (Ellis, 2001; Fischer et al., 2006). The monocyte-macrophages are the phagocytic cells for excellence and are especially abundant in the kidney. Functionally, they are more important in innate immunity and they can be the initiator of activation and regulation of the specific immune response (Uribe et al., 2011). The process of phagocytosis in fish has the same steps as described for mammalian leucocytes, finishing with three mechanisms responsible for the killing of phagocytized microorganisms: the lysosomal enzymes able to digest the ingested pathogens into the phagolysosome, the production of ROS with a rapid and abrupt increase in the rate of oxygen consumption which is known as respiratory burst and is independent of mitochondrial respiration and the production of nitric oxide (NO) and other RNS; all of them showing bactericidal activity (Sharp and Secombes, 1993; Skarmeta et al., 1995). Nonspecific cytotoxic cells (NCCs) in fish are functionally equivalent to natural killer cells (NK) of mammals, forming a heterogeneous population of cells with typical morphological characteristics of monocyte-macrophages, granulocytes and/or lymphocytes (Cuesta et al., 1999) able to kill tumor cells, xenogeneic cells, virus-infected cells and parasites. Regarding the specific immunity, T lymphocytes are responsible for cell-mediated responses, cytokine secretion and also act as helper cells of B lymphocytes (Alberts et al., 2002). The recognition of antigen by T lymphocytes is produced only when are presented properly by the antigen presenting cell (APC) through the major histocompatibility complex of class II proteins or by nucleated cells through the major histocompatibility complex of class I proteins (Danchin et al., 2004). Furthermore, lymphocytes may produce cytokines that activate macrophages after stimulation with an antigen (Scapigliati et al., 2000), showing, therefore, a coordinated and mutual control among the innate and adaptive response.

Therefore, in fish, as in mammals, a network of cytokines and chemokines exist that orchestrate the innate and acquired immune response. But these molecules also regulate many other important biological processes, including cell growth and activation, inflammation, tissue repair, fibrosis and morphogenesis. Cytokines are proteins (usually glycoproteins) with a low molecular weight secreted by immune cells (mainly macrophages and T lymphocytes) in response to pathogens (Secombes et al., 2001). Despite being a heterogeneous group of proteins, they are considered as a protein family from a functional point of view, since not all of them are chemically related but share common properties (Secombes et al., 2001). They have a very short half-life and exhibit pleiotropic attributes (regulate different functions), redundant, synergism, antagonism and induction in cascade. Furthermore, cytokines mediate effector phases in both innate and adaptive immunity (Lu et al., 2008; Pressley et al., 2005). Most of the research has been focused on interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), transforming growth factors (TGFs), migration inhibitory factors (MIFs), the colony stimulating factors (CSFs) and chemokines. In the innate immunity, cytokines are produced mainly by mononuclear phagocytes and so are usually called monokines. However, most of the cytokines involved in adaptive immunity are produced by activated T lymphocytes and these molecules are referred to as lymphokines. Both lymphocytes and mononuclear phagocytes produce other cytokines known as CSFs, which stimulate the proliferation and differentiation of immature leucocytes in the bone marrow. Some other cytokines known as chemokines are chemotactic for specific cell types. Although cytokines are made up of a diverse group of proteins, they share some features such as: (i) they are produced during the effector stages of the innate and adaptive immunity, and regulate the inflammatory and immune response; (ii) their secretion is brief and auto-limited, in general, cytokines are not stored as preformed molecules, and their synthesis is initiated by a new genetic transcription; (iii) a particular cytokine may be produced by many different cellular types; (iv) a particular cytokine may act on different cell types; (v) cytokines usually produce different effects on the same target cell, simultaneously or not; (vi) different cytokines may produce similar effects; (vii) cytokines are usually involved in the synthesis and activity of other cytokines; (viii) cytokines perform their action by binding to specific and high affinity receptors present on the target cell surface; (ix) the expression of cytokine receptors is regulated by specific signals (other cytokines or

even the same one); (x) for many target cells, cytokines act as proliferation factors (Secombes et al., 2001). In fish, cytokines are grouped into growth factors (TGF-β) pro-inflammatory (IL-1β, TNF-α, IL-18, IL-6, etc.), anti-inflammatory (IL-10, IL-19, IL-20, IL-22, etc.) (Laing et al., 1999; Sumathy et al., 1997), anti-viral (IFNs) cytokines (Hansen and La Patra, 2002) and chemokines (CC, CXC, CX_3C, CXCL-8, etc.) (Laing et al., 2002).

4.2. Immunotoxicology produced by metals

A large number of environmental pollutants are capable of suppressing immune responses in fish (Hermann and Kim, 2005; Steinhagen et al., 2004). Unlike mammals, stresses imposed on the fish immune system by environmental pollutants may not always be overtly apparent since stressor agents may directly kill the fish or indirectly aggravate disease states by lowering resistance and allowing the invasion of environmental pathogens (Wester et al., 1994). Toxicological studies conducted in fish preferentially uses field/laboratory *in vivo* exposure because they are more realistic (Cuesta et al., 2011) or fish cell lines (Segner, 1998) to assess the toxicity while the effects on the immune response are still not very well understood. However, *in vitro* tests are gaining traction as alternatives to *in vivo* tests because they are more cost and time effective and have fewer ethical issues and this should be also applied to fish immunotoxicology (Dobrovolskaia et al., 2009). Unfortunately, very few studies have evaluated the immunotoxicological effects of the toxicants *in vitro* because there are very few available immune-related fish cell lines and the use of freshly isolated leucocytes has attracted little interest among researchers.

4.2.1. Cadmium

The effects of Cd on innate immune function represent the best studied area of metal-induced immunotoxicity in fish and are mostly obtained by *in vivo* studies. Thus, Cd exposure failed to alter the seric IgM levels in gilthead seabream (Guardiola et al., 2013a) and medaka (Zelikoff et al., 1995), reduced in common carp (Sövényi and Szakolczai, 1993) and increased or decreased in rainbow trout (Sánchez-Dardon et al., 1999). Regarding the alternative complement activity, this was decreased in gilthead

seabream (Guardiola et al., 2013a) and Nile tilapia (Wu et al., 2007) exposed to Cd. Regarding the cellular immunity, Cd exposure reduced the respiratory burst and increased the phagocytosis in gilthead seabream (Guardiola et al., 2013a) and similar trends were also observed in rainbow trout, dab (*Limanda limanda*), medaka and European sea bass (Bennani et al., 1996; Gagné et al., 2010; Sánchez-Dardon et al., 1999; Zelikoff et al., 1995). Moreover, lymphocyte viability and functions seem to be more affected by Cd exposure than in phagocytic cells (Witeska and Wakulska, 2007). In fact, blastogenic and antibody-production responses of lymphocytes were impaired in sea trout (*Salmo trutta*) (O'Neill, 1981). In addition, some studies have also shown variable effects in the number of circulating leucocytes after Cd-exposure (Drastichova et al., 2004; Shah and Altindag, 2004). Interestingly, one study has shown a decrease in the number of neutrophils in the pronephros and, at the same time, an increase in the thymus of parasitized and Cd-exposed fish indicating that they are mobilized from the kidney to the thymus (Schuwerack et al., 2003).

Few studies have been addressed *in vitro*. Thus, by Cd incubation of leucocytes, the respiratory burst activity of PBLs from European sea bass (Bennani et al., 1996) and common carp (Witeska and Wakulska, 2007) was reduced. Regarding the NCC activity, Cd exposure of head-kidney leucocytes resulted in decreased activity (Viola et al., 1996).

4.2.2. Mercury

Hg and derivatives such as MeHg, are also important contaminants in aquatic environments inducing organ lesions, neurological, haematological and immunological disorders (Sweet and Zelikoff, 2001). First evidences, in rainbow trout, described a decrease in the number of mucous-producing cells and mucus production after exposure to Hg and MeHg, which can be associated to impaired immunity (Lock and van Overbeeke, 1981). Afterwards, serum C-reactive protein was increased in freshwater murrel (*Chana punctatus*) (Ghosh and Bhaattacharya, 1992) and major carp (*Catla catla*) (Paul et al., 1998) by exposure to Hg. However, plasmatic lysozyme of plaice (*Pleuronectes platessa*) was decreased after exposure to sublethal doses of Hg (Fletcher, 1986). In sharp contrast, blue gourami (*Trichogaster trichopterus*) showed increased kidney and plasma lysozyme activity, but at the same time reduced the production of agglutinating specific antibodies after chronic exposure to 0.045 or 0.09 mg Hg^{2+}/L

(Low and Sin, 1998). In addition, in our group, gilthead seabream specimens exposed to waterborne MeHg showed increased serum complement activity as well as head-kidney leucocyte phagocytic activities were increased after 10 and 30 days (Guardiola et al., 2016).

Further evidences have been obtained *in vitro*. For instance, blue gourami lymphocytes incubated with Hg showed increased proliferation at low dosages, which was reversed by higher levels (>0.045 mg/L) (Low and Sin, 1998). In the marine red drum (*Sciaenops ocellatus*), Hg treatment (≤10 μM) produced either activation or inhibition of the PBLs proliferation by high or low dosages, respectively, as well as increases in the Ca^{2+} mobilization and tyrosine phosphorylation of proteins (MacDougal et al., 1996). In the European sea bass, *in vitro* treatment with $HgCl_2$ induced apoptosis in head-kidney macrophages as well as reduced the ROS production and the benefits of macrophage-activating factors (MAF) (Bennani et al., 1996; Sarmento et al., 2004).

4.2.3. Lead

The nonessential metal Pb has detrimental effects on a number of organs and systems, including the immune system (Dietert and Piepenbrink, 2006; Singh et al., 2003). Immunotoxic effects of Pb may result in immunosuppression, rendering an organism more susceptible to infectious diseases or cause inappropriate enhancement of immune response, leading to allergies or autoimmune diseases (Singh et al., 2003). Though the immunotoxicological effects of Pb on the immune response in human or mice are evaluated (Carey et al., 2006; Farrer et al., 2005), very few descriptions are in fish. Thus, first studies observed no alterations in absolute and differential counts of white blood cells in redbelly tilapia (*Tilapia zillii*) exposed to waterborne Pb (at concentrations of 0.3-10 mg/L) (Ghazaly, 1991). By contrast, Adeyemo et al. (2008) observed an increase in lymphocyte percentage, and a decrease in neutrophil contribution accompanied by a PBLs decrease in African catfish (*Clarias gariepinus*) subjected to 25-250 mg/L of Pb for 4 days. On the contrary, Shah and Altindag (2005), and Ates et al. (2008) reported an increase in PBL numbers in Pb-exposed fish.

The scarce *in vitro* data are restricted to common carp leucocytes. Thus, 5.0-100 mg/L of Pb suppressed the activity of phagocytes while no effect was observed at 0.001-1.0 mg/L (Dunier and Siwicki, 1994). In addition, they found that lymphocyte proliferation

was enhanced at 6.2-100 mg/L but completely suppressed at 1.0 mg/L. Pb exposure to common carp PBLs resulted cytotoxic for lymphocytes when exposed to 5-50 µM of Pb, but no effect on the activity of phagocytes was evidenced (Witeska and Wakulska, 2007).

4.2.4. Arsenic

The semimetal or metalloid As is an important environmental toxicant, which has been associated with multitude of animal and human health problems; although, its impact on fish immune system has not been extensively investigated. The antibody production has been reduced by As exposure in several fish species (Datta et al., 2009; Ghosh et al., 2007; Sharifi et al., 2002). Nevertheless, the immunotoxicological effects of As reduced the leucocyte respiratory burst, expression of some immune-relevant genes and disease resistance in zebrafish (Hermann and Kim, 2005; Nayak et al., 2007), in a similar way than in the channel catfish (Datta et al., 2009; Ghosh et al., 2007). In gilthead seabream, exposure to waterborne As resulted in unaltered humoral innate immune responses while head-kidney leucocyte phagocytic, respiratory burst and peroxidase activities were all increased (Guardiola et al., 2013b). In European se bass, waterborne As provoked a significant decrease inthe serum bactericidal activity and head-kidney leucocyte respiratory burst (Cordero et al., 2016 submitted).

In addition, As-exposure *in vitro* produces a selective head-kidney macrophages death (Datta et al., 2009), down-regulates the synthesis of macrophage-derived cytokines such as TNFα and IFN-γ (interferon gamma) (Lage et al., 2006) and decreases the phagocytic activity of macrophages (Ghosh et al., 2006).

Objectives

The present Thesis use fish cell lines or primary cell cultures derived from gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*) for evaluating the *in vitro* toxicological effects of four metals (Cd, Hg, Pb and As). Concretely, the specific objectives of the present work are:

1. Establish and characterize a new brain cell line (DLB-1) from European sea bass and its applications in metal toxicology.

2. Assess the cytotoxicity of metals Cd, Hg, Pb and As in two cell lines (sea bass DLB-1 and seabream SAF-1) and three primary cell cultures [circulating erythrocytes and head-kidney (HKLs) and peripheral blood (PBLs) leucocytes] from gilthead seabream and European sea bass.

3. Evaluate the cellular oxidative stress response of the cell lines and primary cell cultures after metal exposure by measuring ROS levels and/or mRNA transcription of antioxidant enzymes.

4. Evaluate, at gene level, the mechanisms participating on cell protection against metals such as metallothioneins and heat-shock proteins.

5. Elucidate the main cellular death mechanisms induced after metal exposure.

6. Evaluate the immunotoxicological effects of metals on leucocytes from gilthead seabream and European sea bass.

Experimental chapters

Chapter 1

Establishment of a fish brain cell line (DLB-1) and metal neurotoxicology

Abstract

Generation and use of cell lines is of great interest because they are valuable tools for research. In fish, there are not many commercial cell lines and none in the case of European sea bass (*D. labrax*). A continuous cell line from the sea bass brain (DLB-1) has been generated and its usefulness in toxicology tested. DLB-1 cells resemble epithelial cells, express gene markers for glial cells and not for neural cells and result in low transfection numbers. Exposure to metals (Cd, MeHg, Pb or As) produces cytotoxicity, being Cd and Pb the most and less toxic metals, respectively. Moreover, Cd and Pb induced the greatest ROS production. Interestingly, cell cycle analysis of DLB-1 cells exposed to metals showed that exposure to Cd, MeHg or Pb significantly increased the percentage of cells in interphase G0/G1 cells whereas Pb and As increased the percentage of cells in S phase and almost abrogated the G2/M phase. Moreover, all the metals induce apoptosis cell death as indicated by sub-Go/G1 population in the cell cycle analysis and annexin binding. Finally, exposure of DLB-1 cells to metals also produce significant alterations at gene expression level that confirm the above results such as down-regulation of anti-apoptotic (*bcl2*) and antioxidant system (*sod* and *cat*) gene transcription. This is the first study in which metal cytotoxicity has been evaluated in a fish brain cell line and results seem to support that DLB-1 cells are suitable for toxicological studies.

1. Introduction

Fish cell lines are increasingly becoming more important as research resources, both to gain basic knowledge and to obtain tools that can be used in fish species of interest in aquaculture. In contrast to mammalian cells, fish cells are easier to handle since they have a wider temperature range for cultivation and lower metabolic rates and therefore their maintenance and manipulation requires lower care and conditions (Wolf, 1976). Though the use of commercial or deposited fish cell lines is usually preferred it is also common the need to develop and establish new cell lines for different purposes. Currently, there are around 300 fish cell lines, and around half of them are commercially available. Those cell lines are derived from marine (i.e. SAF-1, SPH, GF-1, CRF-1, etc.) or freshwater fish species (i.e. BF-2, WSF, GFM, PSP and, more recently, zebrafish-derived cell lines). Specially, great interest due to their use in aquaculture settings have received fish cell lines derived from salmonid (i.e. RTG-2, CHSE-124) or cyprinid (i.e. EPC, MG-3, RG-1) species (Gillies et al., 1986; Lakra et al., 2011; Segner, 1998).

In general, most of the fish cell lines are generated from normal tissues, mainly fins and embryos, however, there are not many cell lines obtained from fish brain even considering that this tissue conserves high rates of neurogenesis and regeneration after injury (Servili et al., 2009; Zupanc and Clint, 2003). Thus, there are some studies conducted with short- to long-term cultures or generation of brain-derived cell lines from several fish including catla (CB), groupers (GB, GBC1, GBC4, RGB), barramundi (BB), tilapia (TB2), European sea bass (SBB-W1), Asian sea bass (ASBB), Japanese flounder (POBC), half-smooth tongue sole (CSH), Chinese perch (CPB) or American eel (eelB) among others (Ahmed et al., 2009; Bloch et al., 2015; Chen et al., 2010; Chi et al., 2005; Fu et al., 2015; Hasoon et al., 2011; Ku et al., 2009; Lai et al., 2001, 2003; Servili et al., 2009; Wang et al., 2010; Wen et al., 2008a, b., Zheng et al., 2015).

Early works with fish cell lines were mainly initiated for virological studies (Wolf, 1988) followed by toxicology and ecotoxicology applications (Bols et al., 2001, 2005; Schirmer, 2006). In fact, many cytotoxicity studies using cell lines have positively related the *in vitro* and *in vivo* EC_{50} (effective concentration causing 50% death in a population) values upon exposure to different contaminants, demonstrating the value of

using fish cell lines in this field of research (Castaño et al., 1996; Fent, 2001; Segner, 2004; Vega et al., 1996). Among these toxicants, metals derived from industrial and mining can seriously affect aquatic communities (Uysal et al., 2009). Metals are not biologically or chemically degraded but may be bioaccumulated in the food chain since they are found at high concentrations in fish living in contaminated waters (Gibbs and Miskiewicz, 1995; Tariq et al., 1993). This is also of relevance in the case of cultured fish which are fed diets containing fish oils and meals from the wild. The accumulation of toxicants may have a negative impact not only for fishes but also for their consumers (Fernandes et al., 2008). In fact, fish are known to be the greatest source of toxic trace elements (Hg, Al, Se, Cd, Pb, As, Cu, Cr, Fe, Mn, Mb, Va and Zn) for humans (EFSA, 2005; Minganti et al., 2010). Thus, how metals are accumulated and how they can affect cultured fish as well as the levels they transfer to humans is important information for the aquaculture industry in order to develop efficient restrain measures. Among the effects of metals, it is widely known that some of them such as Hg, especially in the form of MeHg, Cd, Cu, Al, Pb or Sn, as well as other many toxicants, are neurotoxic (Bradbury et al., 2008). Unfortunately, to date there are no studies using fish brain-derived cell lines to study neurotoxicity in fish.

Among marine fish species, European sea bass (*D. labrax*) is a very important fish for the aquaculture industry and one of the most farmed in the Mediterranean area whereby there are no commercial cell lines. To the best of our knowledge, European sea bass continuous cell line (DLEC) (Buonocore et al., 2006) and a long term culture cells from brain (SBB-W1) (Servili et al., 2009) have been described. DLEC cell line, derived from early embryos, are fibroblast-like and adherent cells and have shown to be useful in immunological (Casani et al., 2009) and toxicological (Rocco et al., 2014) studies. By contrast, SSB-W1 cells resemble neural stem cells (Servili et al., 2009) and unfortunately no other study has been performed since their characterization. Therefore, the aim of this study was to generate and to characterize a continuous cell line from the brain of European sea bass (DLB-1). Furthermore, its applicability to general toxicology, and neurotoxicology in particular, was established. For this purpose, DLB-1 cells were exposed to metals Cd, Pb, Hg or As and the cytotoxicity, oxidative stress and cell death were evaluated by functional and transcriptional studies.

2. Materials and methods

2.1. Generation and culture of the DLB-1 cell line

One asymptomatic European sea bass (*D. labrax*) fish specimen (83 g of body weight) was bred and supplied by the *Instituto Español de Oceanografía* (IEO; Mazarrón, Murcia, Spain) and kept in seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. Fish was anaesthetised with benzocaine (4% in acetone) (Sigma-Aldrich) and bled from the caudal vein to avoid excessive tissue contamination with blood cells. Fish was dissected under sterile conditions and brain fragments were obtained using sterile forceps and scalpels. Fragments were disposed into a tissue culture flask (Nunc) using L-15 Leibowitz medium (Thermo Fisher Scientific) supplemented with 15% fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 100 µg/mL streptomycin (Thermo Fisher Scientific), 100 U/mL penicillin (Thermo Fisher Scientific) and 100 mM HEPES (Thermo Fisher Scientific). Brain fragments were cultured at 25ºC in an incubator with an atmosphere with 85% relative humidity. The medium osmolarity was measured by an osmometer (Röebling) and adjusted to the osmolarity of fish serum (355 mOsm/kg). Explants were checked every day by an inverted contrast phase microscope (Nikon Eclipse TE 2000-U;Nikon) and subcultured upon reaching confluence by standard trypsinization methods using 0.25% trypsin solution containing 0.53 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich). After a first passage (P1), to facilitate cell immortalization, culture was inoculated with filtered supernatants from the SSN-1 cell line that is persistently infected with the snakehead (*Ophicephalus striatus*) retrovirus (SnRV) (Family *Retroviridae*, genus *Orthoretrovirinae*) (Frerichs et al., 1996). Cultures were continued and time between passages was gradually decreasing from around 1 month at the beginning until 7-10 days after 25-30 passages. Since then, the obtained DLB-1 brain cell line has more than 90 passages and is subcultured by passaging every week with a 1:4 dilution.

2.2. Characterization of the DLB-1 cell line

All the techniques used to characterize the DLB-1 cell line were applied to cells from 60 passages onwards.

2.2.1. Microscopical study

Evolution of the cultures from the explant to the cell line consolidation was routinely followed by an inverted phase contrast microscope (Nikon Eclipse TE 2000-U; Nikon) and photographs were obtained with a digital camera (Nikon DS-5M; Nikon). For further microscopical studies, sterile glass coverslips were placed in 6-well cell culture plates (Nunc) and 7.5×10^4 DLB-1 cells/well were cultured on them for two days. For light microscopy, coverslips with DLB-1 cells were processed for standard Giemsa, toluidine blue and haematoxylin-eosin (HE) staining methods being examined under a light microscope (Leica 6000B) being the images acquired with a Leica DFC280 digital camera.

For scanning electron microscopy (SEM), coverslips with cultured adhered cells were washed, fixed in 3% (v/v) glutaraldehyde for 30 min and post-fixed in 1% OsO_4 for 1 h. Afterwards, samples were dehydrated in acetone, critical-point dried, sputter coated with gold and examined with a Jeol 6.100 scanning electron microscope.

2.2.2. DLB-1 cell growth curve and doubling time

To obtain the DLB-1 cell line growth curves $1\text{-}50\times10^3$ DLB-1 cells per well were seeded into 96 well-plates (Nunc) and incubated from 0 to 7 days at 25ºC. The cell viability was evaluated every day by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) colorimetric assay based on the reduction of the yellow soluble tetrazolium salt into a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase (Mosmann, 1983). For this, DLB-1 cells were washed and incubated with 200 µL/well of culture medium containing 1 mg/mL of MTT. After 4 h of incubation at 25ºC, the wells were washed, the formazan solubilized and the absorbance at 570 nm and 690 nm was determined in a microplate reader (BMG Labtech). Blanks were consisted of wells without cells. In order to relate absorbance with DLB-1 cell numbers, serial dilutions ($1\text{-}200\times10^3$) of DLB-1 cells per well were also seed, incubated for 4 h to adhere and then the MTT test performed as above.

To calculate the doubling time (Dt) we used the data from the linear part of the growth curves and applied the following formula (Roth, 2006 http://www.doubling-time.com/compute.php):

Dt = duration×log (2)/[log(final concentration)-log(initial concentration)].

2.2.3. DLB-1 characterization by gene expression

We evaluated the expression of snakehead retrovirus polymerase gene (*pol*) by conventional PCR (Table I). Thereby, DLB-1 cells from 5 and 70 passages were washed and TRIzol Reagent (Thermo Fisher Scientific) was added to the wells in order to extract the total RNA. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with random hexamers following the manufacturer's instructions. The cDNA was then used in a PCR amplification round with specific primers (Table I) using Taq polymerase (Thermo Fisher Scientific) and the amplification performed in a Master Cycler Gradient PCR as follows: 95ºC for 5 min, 40 cycles of 95ºC for 45 s, 58ºC for 45 s, 72ºC for 45 s, and followed by 72ºC for 10 min. PCR products were separated on a 2% agarose gel containing GelRed™ (Biotium) and visualised under UV light. Photographs were taken with the GL 100 Imaging system (Kodak). PCRs using cDNA obtained from gilthead seabream (*S. aurata*) SAF-1 cell line, striped snakehead (*O. striatus*) SSN-1 cell line and European sea bass head-kidney leucocytes were used to confirm the results.

Furthermore, in order to characterize the potential cell type of the DLB-1 cells the expression of neuronal [RNA binding protein fox-1 homolog 3-like (*rbfox3*) and microtubule-associated protein 2-like (*map2*)] or glial [actin binding protein coronin-1a (*coro1a*) and glial fibrillary acidic protein (*gfap*)] cell gene markers was evaluated by real-time PCR (RT-PCR). cDNA from DLB-1 cells at 70 passage (see above) and brain of naïve European sea bass (as positive control) were used to evaluate the expression for both cell type markers by qPCR, which was performed with an ABI PRISM 7500 instrument (Thermo Fisher Scientific) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 µL of 2 × SYBR Green supermix, 5 µL of primers (0.6 mM each) and 5 µL of cDNA template) were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1µ (*ef1a*) RNA content in each sample. Gene names follow the accepted nomenclature for zebrafish (http://zfin.org/). Genes and primers are shown in Table I. In all cases, each PCR was performed with duplicate samples.

Table 1. Oligonucleotide primers used for conventional and real-time PCR. Sequences were retrieved from GenBank and European seabass Genome (http://seabass.mpipz.mpg.de).

Group	Gene	Abbreviation	Acc. number	Primer sequence (5′→3′)
Snakehead retrovirus	Polymerase	*pol*	SRU26458	TGGTACCCATGGATACAGGTACCTCA TGTCAGACATGGCCTGTACTTTAGCAGC
Neuronal markers	Microtubule-associated protein 2-like	*map2*	DLAgn_00049570	AGGCGGTAACGTGCGTATAG GAGACTGCCGGAGGATGATA
	RNA binding protein fox-1 homolog 3-like	*rbfox3*	DLAgn_00230330	AGGCGGTAACGTGCGTATAG GAGACTGCCGGAGGATGATA
Glial cell markers	Glial fibrillary acidic protein	*gfap*	DLAgn_00193250	CCAGATCCAAGTGGTGACCT TGGTGATCCTGCTCTCCTCT
	Actin binding protein coronin-1a	*coro1a*	DLAgn_00193020	AGCCGATGAATGGTTTCAAG CAGCTCCTCTAACGCTGCTT
Cellular protection	Metallothionein	*mt*	AF199014	GCACCACCTGCAAGAAGACT AGCTGGTGTCGCACGTCT
Oxidative stress	Catalase	*cat*	FJ860003	GAGGTTTGCCTGATGGCTAC TGCAGTAGAAACGCTCACA
	Cu/Zn superoxide dismutase	*sod*	FJ860004	TGTTGGAGACCTGGGAGATG ATTGGGCCTGTGAGAGTGAG
Apoptosis	B-cell lymphoma 2	*bcl2*	DLAgn_00005980	GACTGTACCAGCCGGACTTC GTCCCGGAACAGTTCGTCTA
House-keeping	Elongation factor 1-alpha	*ef1a*	AJ866727	CGTTGGCTTCAACATCAAGA GAAGTTGTCTGCTCCCTTGG

2.2.4. Transfection with GFP reporter gene

The vector pEGFP-N3 (Clontech) expresses green fluorescent protein (GFP) under the control of the human cytomegalovirus (CMV) promoter. Plasmid DNA of this vector was prepared according to the supplier's instructions. Lipofectamine® LTX with Plus™ Reagent (Thermo Fisher Scientific) was used for transfection according to the supplier over 80–90% confluent DLB-1 cells seed in 12 well-plates. Green fluorescence was observed up to 24 h under a fluorescence microscope equipped (Nikon Eclipse TE 2000-U; Nikon) with a mercury lamp and photographs were taken by a digital camera.

2.3. Evaluation of metal cytotoxicity

2.3.1. Metal exposure

Different salts of the metals (Sigma-Aldrich) were used: Cd chloride ($CdCl_2$), MeHg (II) chloride [CH_3HgCl (MeHg)], lead (II) nitrate ($Pb(NO_3)_2$) and trioxide As (As_2O_3). DLB-1 cells were plated in 96-well plates at 2.5×10^4 cells/well, cultured overnight and washed with fresh medium. Then, 180 µL/well of fresh culture medium plus 20 µL/well of water (controls) or metal were daily prepared to make the following final

concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 mM of Cd, MeHg or As and 0.25, 0.5, 1, 2, 2.5, 3 and 4 mM of Pb. Cells were exposed for 24 h at 25ºC in the incubator.

2.3.2. Cytotoxicity assays

DLB-1 cells viability was determined after metal exposure by MTT (see above) and neutral red (NR) uptake tests (Babich and Borenfreund, 1991). For NR uptake test, after 24 h of metal exposure, cells were washed with 10 mM phosphate saline buffer (PBS) and 100 µL/well of 0.33% neutral red solution (Sigma-Aldrich) added to the DLB-1 cell cultures. After incubation (3 h at 25ºC) the cells were washed and fixed with 1% acetic acid and 50% absolute ethanol and the absorbance was measured at 540 nm and 690 nm in a microplate reader. Blanks consisted of wells without cells.

Cytotoxicity experiments were repeated three times, with 5 replicates for each condition. The viability in each sample was determined according to the controls or unexposed cells (100% viability). For each method, cell viability data and the logarithm of metal concentrations were represented and fitted with the recursive equation (a regression model). Fitted curves always showed r^2 values higher than 0.95 which are therefore the only ones presented in the graphs. The effective concentration producing the 50% cell death (EC_{50}) was determined using Sigma plot software. According to the MTT method, the EC_{50} dosages were used in other assays.

2.3.3. ROS production

Dihydrorhodamine 123 (DHR 123; Thermo Fisher Scientific) was used to measure ROS production (Henderson and Chappell, 1993) by metal-exposed DLB-1 cells. DHR 123 is able to diffuse through cell membranes and when oxidized by ROS (mainly by hydrogen peroxide) becomes green fluorescent rhodamine 123 which is sequestered into the mitochondria. After 24 h of exposure to EC_{50} dosages of each metal, DLB-1 cells were detached by trypsin/EDTA solution (see above), resuspended in 200 µL of fresh medium with 5 µM of DHR 123 and incubated for 30 min at 25ºC. The samples were then analysed in a FACScalibur flow cytometer (Becton Dickinson) to ascertain the extent of ROS production as indicated by the mean green fluorescence (FL1) intensity for each treatment. In each case, 10,000 events were acquired. The experiment was performed twice with duplicated samples.

2.3.4. Evaluation of the DLB-1 cell cycle

After 24 h of exposure to the EC_{50} dosages of each metal DLB-1 cells were detached by trypsin/EDTA solution (see above) and washed twice with PBS. Cells were resuspended in 200 µL of PBS and 1 mL of a 70% ethanol solution was added dropwise while stirring. After 30 min of incubation, cells were washed and resuspended in 800 µL of PBS. Finally, 100 µL of RNAse (1 mg/mL; Thermo Fisher Scientific) and 100 µL of PI (400 µg/mL; Sigma-Aldrich) were added and incubated at 25ºC for 30 min in the dark. Samples were acquired by a FACScalibur flow cytometer and the cell cycle analysis was performed on 30,000 events using the ModFit LT™ software (Verity Software House). The experiment was performed twice with duplicated samples.

2.3.5. Evaluation of cell death mechanisms: Apoptosis and necrosis

To investigate cell death mechanisms induced by metals we used the Annexin-V-FLUOS staining kit (Roche) that discriminates between apoptosis (Annexin V, green fluorescence) and necrosis (PI, red fluorescence) cell death. To do this, DLB-1 cells exposed to the EC_{50} dosages of each metal and their unexposed controls were collected by trypsinization (see above) and washed twice with PBS. DLB-1 cells were incubated with Annexin-V-FLUOS staining kit solution at room temperature in the dark for 15 min. Then, samples were analysed by a FACScalibur flow cytometer and the green (by Annexin-V-FLUOS) and red (by PI) fluorescence intensities were analysed. In each case, 10,000 events were acquired. The experiment was performed twice with duplicated samples.

2.3.6. Effects of metal-exposure at gene level

After DLB-1 metal exposure to the EC_{50} of metals, cDNA was synthesized and qPCR was performed as above. The expression of the genes included those involved in: cellular oxidative stress (*sod* and *cat*), cellular protection to metals (*mt*) and cell apoptosis (*bcl2*).

2.6. Statistical analysis

Data of the cytotoxic effects are presented for each metal and colorimetric method using the fitted curves. The results of ROS production are expressed as mean ± standard error, SE. Gene expression is expressed as fold change respect to the control cells. Data were statistically analysed by one-way analysis of variance (ANOVA; $P<0.05$) followed by a post-hoc Tukey test to determine differences among treatments. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. A nonparametric Kruskal-Wallis test was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPPS 20.0 software (SPSS).

3. Results

3.1. Morphological characteristics of the DLB-1 cell line

Primary cell cultures from brain tissue of European sea bass were carried out by using explant culture method and transformation with a fish retrovirus. Cells started to adhere to the culture surface surrounding the explant since the first days until reached confluence within a month (Figure 9A). At very early passages the cell showed a heterogeneous shape and varying morphologies from small cells with very large thin processes to round outlined cells (Figure 9B). Cultures of adherent cells seemed to be progressively more homogenous after subcultures, and cellular shape, mostly epithelioid-like cells, varied depending on the culture cell density with the presence of many start-like cells resembling astrocytes (Figure 9B-E). This was particularly evident in DLB-1 cells stained with HE (data not shown), toluidine blue (Figure 9E) or Giemsa (Figure 9F) which showed a slightly stained cytoplasm provided of a round euchromatinic nucleus with two prominent nucleoli and a wide diversity in shape cell processes. SEM analysis (Figure 9G-H) confirmed the irregular cell outlines as determined by the presence of several cell processes variable in size and shape, and allowed to observe the existence of a varied number of delicate thin branched processes that seemed to firmly adhere to the culture surface. Some of the cells showed an elongated prominent thick part of the cytoplasm and where provided of very few delicate thin long processes (Figure 9I).

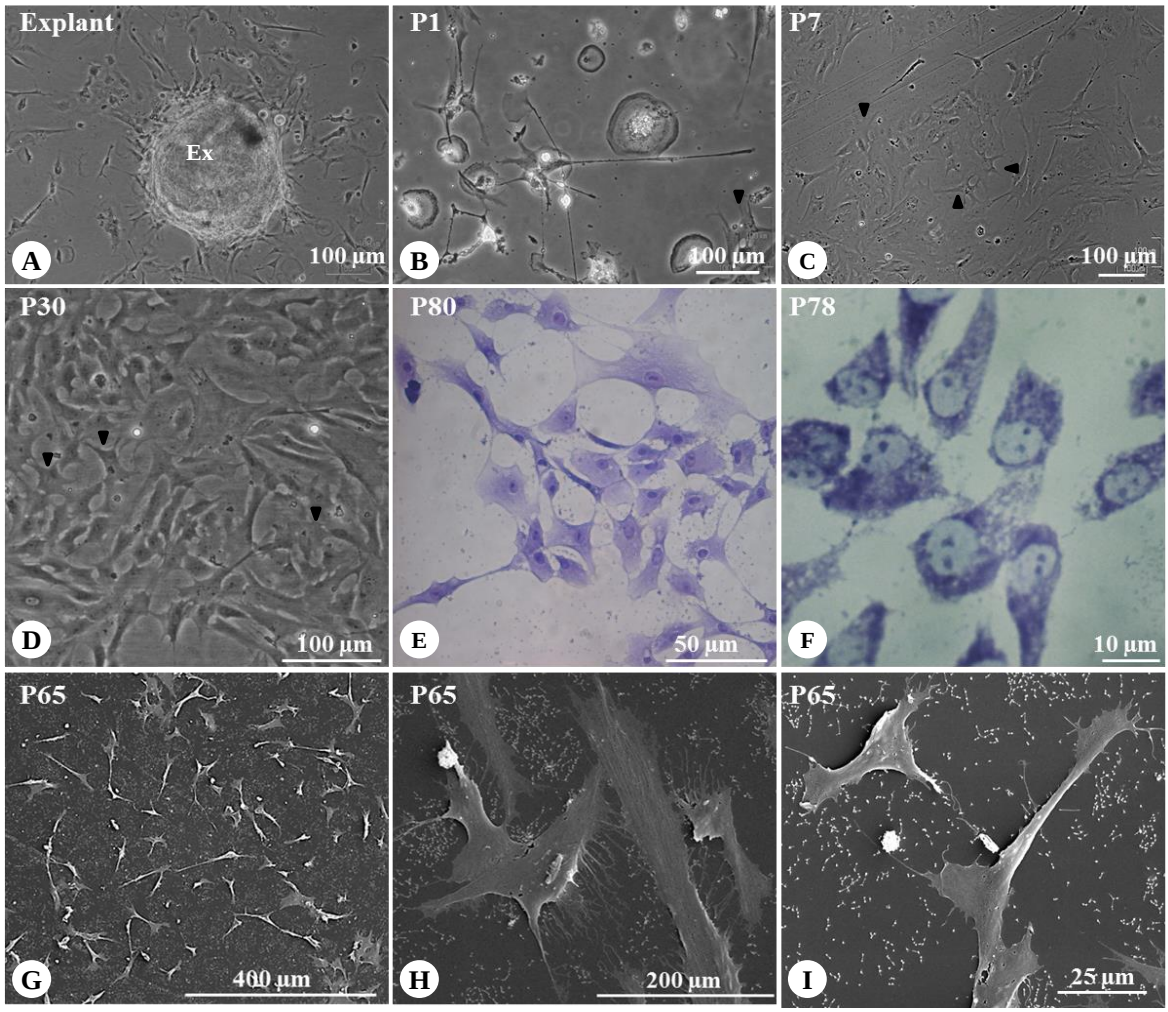

Figure 9. Morphological features of the DLB-1 cells. A-D. Phase contrast microscopy. E. Toluidine blue staining. F. Giemsa staining. G-I. Scanning electron microscopy (SEM). Ex, explant; Arrowhead, star-like cells; P, passages number.

3.2. DLB-1 cell line growth curve and doubling time

The relation between MTT absorbance and cell number followed a linear regression curve in the assayed conditions (Figure 10A). DLB-1 cells exhibited, depending on the initial concentration, the typical lag, exponential, stationary and death phases of a cell line growth curve (Figure 10B). Based on these growth curves, we selected the optimal concentration of 25×10^3cells per well in order to carry out the rest of the experiments because it showed very good values of MTT absorbance in the exponential phase within 1 or 2 days.

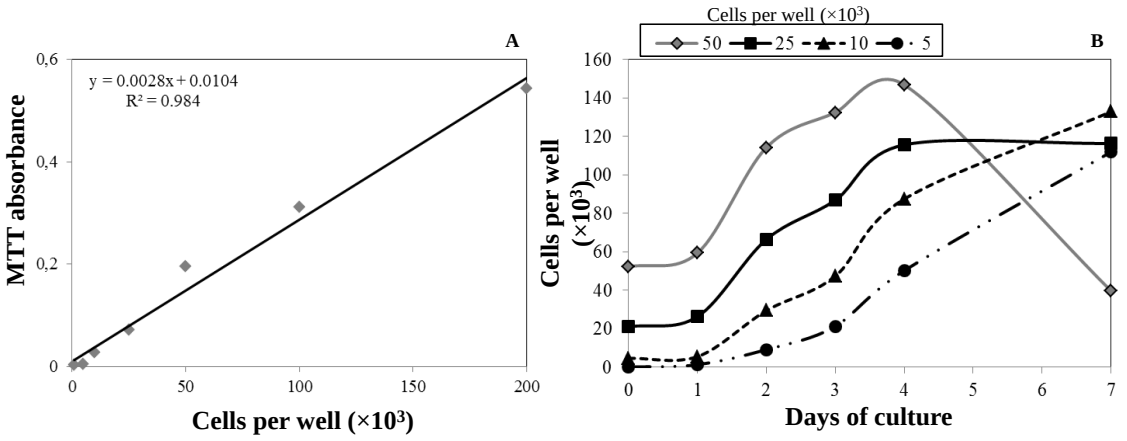

Figure 10. Cell growth curve of DLB-1 cells cultured at 25ºC. A. Relation between MTT absorbance and the cell number. Cells were plated at 1-200×10^3 into wells, allowed to adhere and cell viability was determined by the MTT assay. The equation and R^2 value are presented. B. DLB-1 growth curve expressed as cell number vs incubation time. Cells were plated at 1-50×10^3 into wells and cell viability was determined by the MTT assay from day 0 to 4 and at day 7. Data are representative of two independent experiments.

According to the growth curves, we obtained the maximum slope to calculate the doubling time (Dt). Thus, the DLB-1 cell line Dt was of 22.41 ± 4.5 h.

3.3. DLB-1 cells are infected with retrovirus and express glial cell markers

First, we checked whether the DLB-1 cells were infected with the added SnRV. Thus, we found by PCR that the cells were positive for SnRV *pol* gene after 5 and 70 passages (Figure 11A) as the positive control (SSN-1 supernatant). In contrast, sea bass head-kidney leucocytes (HKLs) or the gilthead seabream SAF-1 cell line were both negative for the retrovirus.

In addition, we evaluated the gene expression of two neuron (*rbfox3* and *map2*) and two glial (*gfap* and *coro1a*) cell markers (Figure 11B). All the genes were expressed in naïve sea bass brain tissue whilst only those expressed in glial cells were detected in the DLB-1 cell line indicating that this cell line is from glial origin and not neuronal.

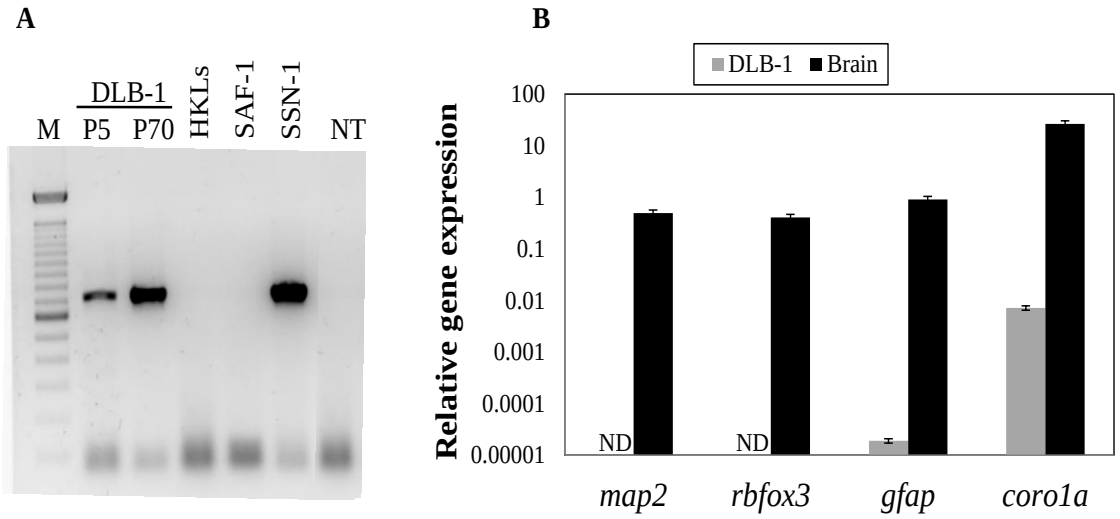

Figure 11. Expression of retrovirus and cell marker genes in the DLB-1 cell line. **A.** Detection of the SnRV *pol* gene in the DLB-1 cells after passages (P) 5 and 70, in the European sea bass head-kidney leucocytes (HKLs), in the gilthead seabream SAF-1 or striped snakehead SSN-1 (positive control) cell lines. NT, no template. **B.** Transcription of neuronal [microtubule-associated protein 2-like (*map2*) and RNA binding protein fox-1 homolog 3-like (*rbfox3*)] or glial [glial fibrillary acidic protein (*gfap*) and actin binding protein coronin-1a (*coro1a*)] cell gene markers evaluated by real-time PCR in DLB-1 cells at passage 70 and in brain of naïve sea bass as positive control. Data are expressed as relative to the house-keeping gene expression (*ef1a*). ND, not detected.

3.4. DLB-1 cell line is slightly transfected

DLB-1 cells were transfected with 10 µg pEGFP-N3 vector using liposomes and green fluorescent signals were observed after 48 h of transfection (**Supplementary FigureI.SI**). The transfection efficiency was lower to 10% indicating that reporter gene GFP could be expressed in DLB-1 cell line.

3.5. Cytotoxicity assays

DLB-1 cells exposed to metals showed cytotoxicity as evidenced by the decrease in viability observed by both MTT and NR assays (**Figure 12**). For each method, cell viability data and metal concentrations were represented. The fitted curves, which were the only ones presented, always showed r^2 values higher than 0.95. The curves show

dose-dependent cytotoxicity from which reliable EC_{50} values could be calculated (Figure 12 and Table 2). NR method was more sensible in the case of Cd and MeHg while the MTT assay was for Pb and As. This fact makes a significant difference since the cytotoxicity is in the order of Cd>MeHg>As>>>Pb for NR method but in the order of Cd>As>MeHg>>>Pb for the MTT assay (Table 2).

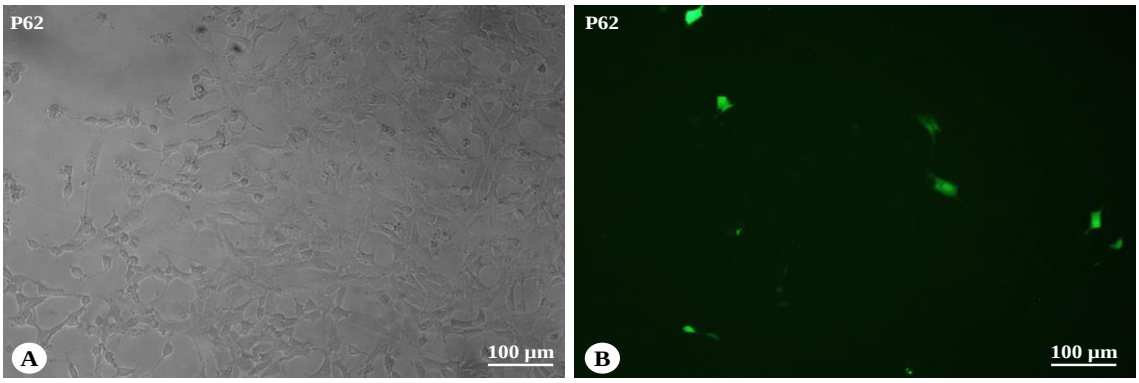

Figure I.SI. DLB-1 cells show low transfection efficiency. DLB-1 cell cultures were transfected with the vector pEGFP-N3 and after 24 h the transfection was visualized. A. Phase contrast. B. Fluorescence microscopy. P, passages number.

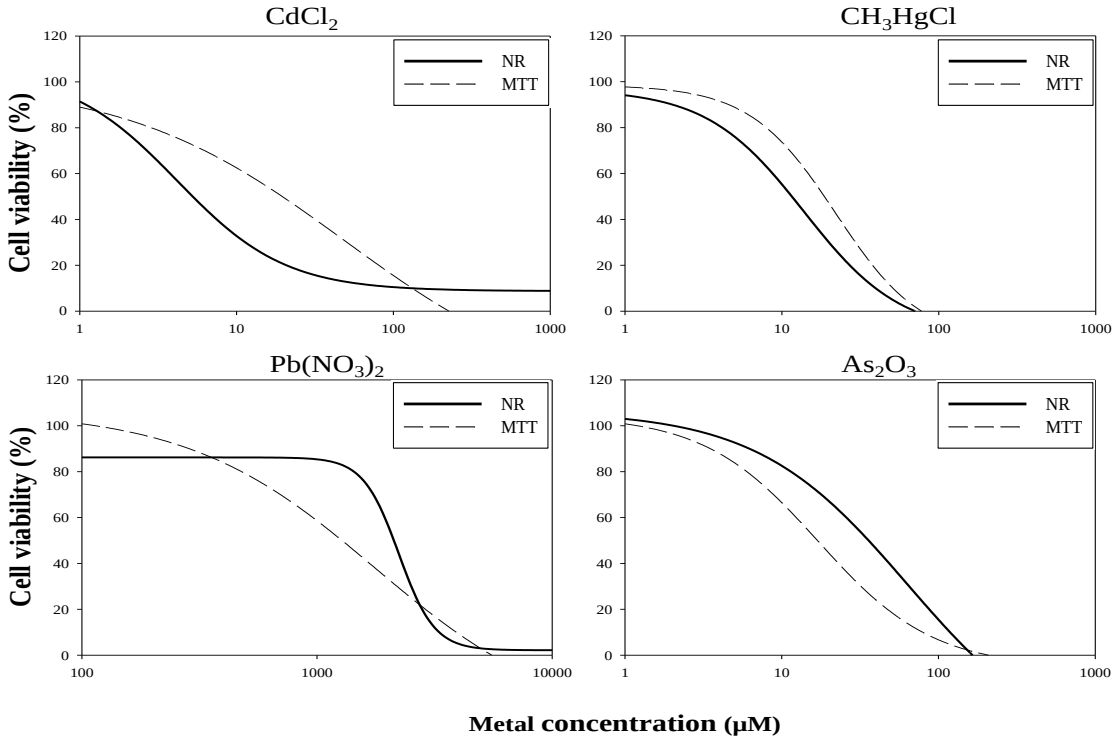

Figure 12. Cytotoxicity curves of DLB-1 cells exposed to different metals for 24 h. Lines represent the fitted curve for each method: NR, neutral red, MTT, yellow soluble tetrazolium salt.

Table 2.Values of r^2 and EC_{50} (mM) after exposure of DLB-1 cells to metals for 24 h. NR, neutral red; MTT, tetrazolium salt.

Metals	NR		MTT	
	r^2	EC_{50}	r^2	EC_{50}
$CdCl_2$	0.95	0.004	0.98	0.015
CH_3HgCl	0.95	0.013	0.96	0.020
$Pb(NO_3)_2$	0.98	1.6	0.97	1.5
As_2O_3	0.99	0.03	0.99	0.018

3.6. ROS production and antioxidant system are disturbed by metals

ROS production, measured by the DHR 123 method (Figure 13A), showed that DLB-1 cells exposed to the EC_{50} of Cd or Pb for 24 h significantly increase the production of ROS compared to the controls ($P<0.05$) whilst exposure to MeHg or As failed to alter the ROS production in DLB-1 cells (Figure 13B). In addition, we also evaluated this phenomenon by the expression of genes related to the generation (*sod*) and elimination (*cat*) of ROS. Interestingly, *sod* transcription (Figure 13C) was unmodified upon metal exposure, while *cat* gene expression was significantly down-regulated upon MeHg, Pb or As exposure (Figure 13D). In addition, MTs are metal-binding proteins with ROS scavenging functions (Ruttkay-Nedecky et al., 2013). Thus, we found that DLB-1 cells exposed to EC_{50} of MeHg and As for 24 h strongly up-regulated *mt* transcription, which coincides with the lowest ROS production, but Cd and Pb failed to alter it to a significant extent (Figure 13E).

3.7. Cell cycle is altered by metal exposure

First, cell cycle analysis indicates that DLB-1 cells show a typical diploid cell shape (Figure 14A). DLB-1 cells exposure to Cd, MeHg or Pb provoked a significant increment in the percentage of cells in phase G0/G1 while As reduced it (Figure 14B). Regarding the S phase or DNA duplication, MeHg, Pb and As significantly increase the percentage

of cells at S phase. However, the mitosis of G2/M phase was significantly decreased by all the tested metals (Figure 14B).

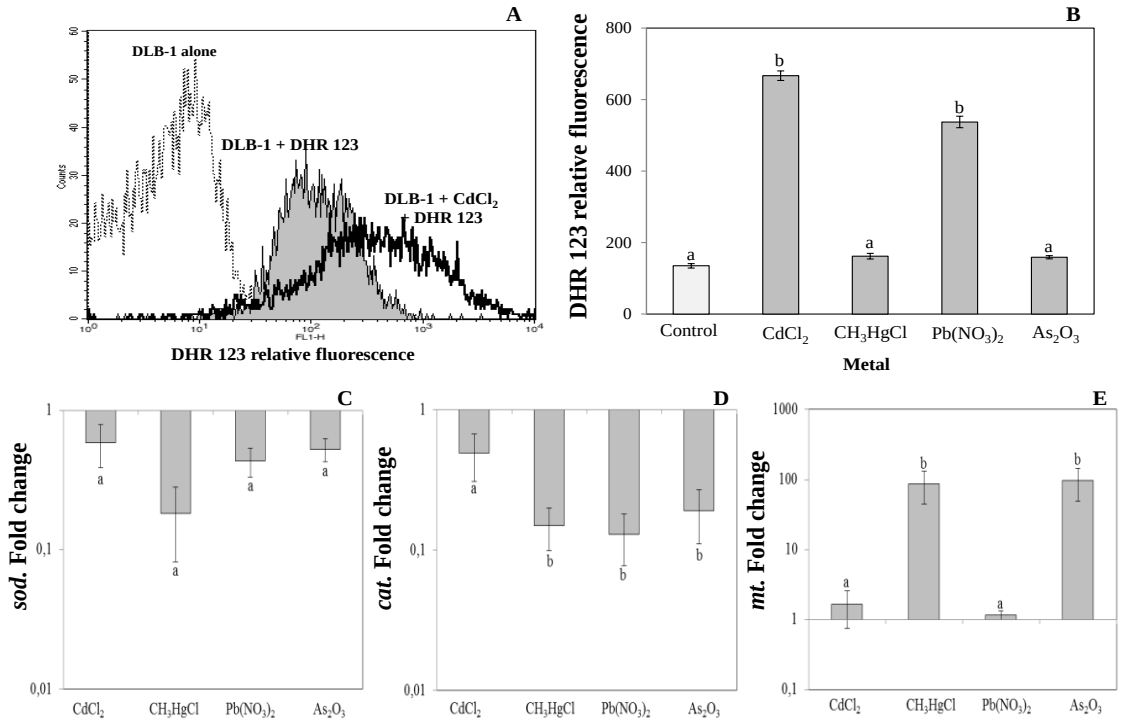

Figure 13. Metal exposure induces overproduction of ROS and impairs antioxidant system in DLB-1 cells. A. Representative histogram of DHR 123, as measure of ROS production, in unstained cells and in stained and unexposed or Cd-exposed DLB-1 cells. B. DHR 123 fluorescence intensity of DLB-1 cells after exposure to EC_{50} of metals for 24 h. C. Expression of *sod* gene. D. Expression of *cat* gene. E. Expression of *mt* gene. Gene expressions are expressed as fold change with respect to control cells. Bars represent the mean ± SEM (n=2 separate experiments). Statistical analysis was performed by ANOVA and post-hoc Tukey test ($P \leq 0.05$). Different letters denote statistically significant differences among groups (control group always contains a).

3.8. Apoptosis cell death is induced by metals

We also evaluated whether metals provoked the cell death by necrosis or apoptosis pathways (Figure 15). Our data showed that apoptosis was the only cell death mechanisms used by the metals as indicated by the double staining with annexin and PI (Figure 15A-C) as well as the sup-G0/G1 population in the cell cycle analysis (Figure

I5D-E). Though both techniques differed in the absolute percentages and therefore in sensitivity in the two cases Cd and As were the metals producing the highest apoptotic rates followed by MeHg and Pb. At gene level, *bcl2* gene expression was down-regulated by MeHg, Pb and As (Figure I5F), confirming the apoptosis cell death by the other two methods.

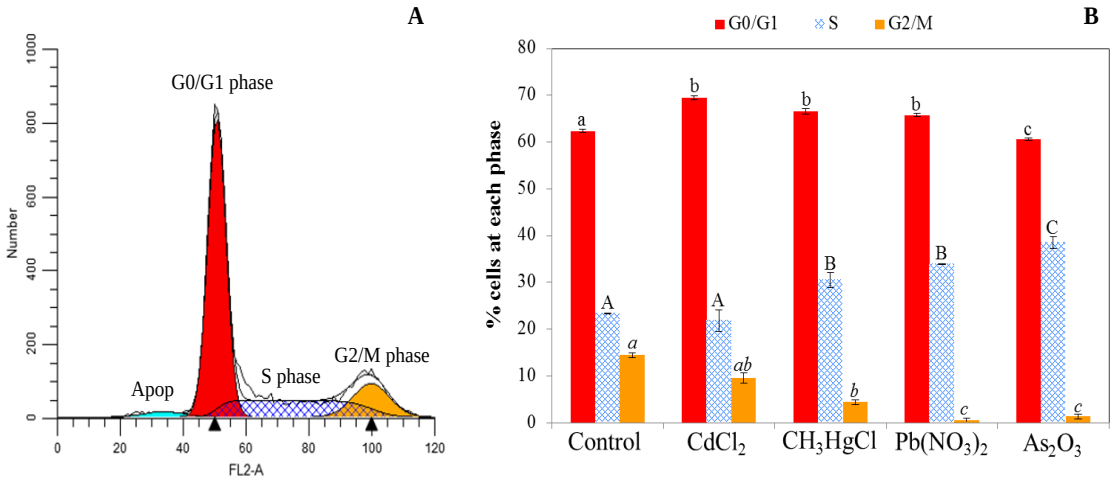

Figure I4. Cell cycle and metal protection is affected by metal exposure in DLB-1 cells. A. Analysis of control DLB-1 cells cycle by flow cytometry showing cells in resting G0/G1, S and G2/M phases and sub-(G0/G1) or apoptotic cells. B. Evaluation of the cell cycle analysis in DLB-1 cells exposed for 24 h to EC_{50} of metals. Bars represent the mean ± SEM (n=2 separate experiments). Different letters denote statistically significant differences among groups (control group always contains a).

4. Discussion

The advantages of fish cell lines with respect to mammalian cells are that they are easily standardized handled with relatively low variability, more convenient, and less laborious to use (Rocco et al., 2014) and therefore merits further generation and application. Fish cell lines derived from brain are very scarce and there is no continuous cell lines from the brain of European sea bass, the main objective in this study. The usefulness of DLB-1 cells in fish immunology and virology is also under evaluation in our laboratory (Valero et al, 2015b), however, we will focus in this manuscript in its

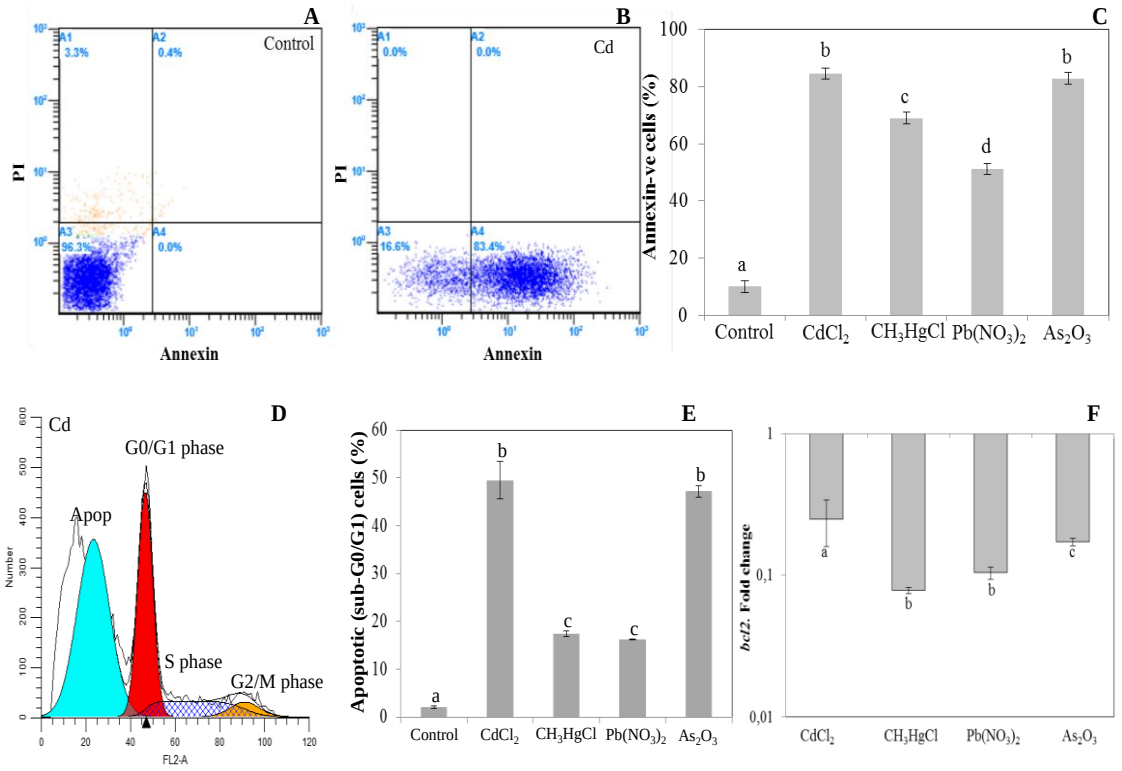

Figure 15. Apoptosis is induced by metals in DLB-1 cells. A. Double staining with Annexin (apoptosis) and PI (necrosis) of unexposed (control) DLB-1 cells. B. Annexin/PI staining of DLB-1 cells exposed to EC_{50} of Cd for 24 h. C. Percentage of apoptotic DLB-1 cells after exposure to EC_{50} of metals for 24 h. D. Cell cycle analysis of DLB-1 cells exposed to EC_{50} of Cd for 24 h showing increased population of apoptotic or sub-(G0/G1) cells. E. Percentage of the sub-(G0/G1) DLB-1 cells population after exposure to EC_{50} of metals for 24 h. F. Transcription of B-cell lymphoma 2 (*bcl2*) gene expressed as fold change with respect to control cells. Bars represent the mean ± SEM from 2 independent experiments. Different letters denote statistically significant differences among groups (control group always contains a).

characterization and application in Toxicology. We have obtained an established a cell line from the European sea bass brain (DLB-1) after transformation with a fish retrovirus (SnRV) (Frerichs et al., 1996). This method increases the success of cell immortalization and should be further used in fish. To our knowledge, this is the first time a retrovirus is used to obtain fish brain cell lines though it is common to use viral oncogenes such as simian virus-40 (SV40) large T-antigen in mammals but no in fish cells (Luque et al., 2014). Nowadays, DLB-1 cells show a rapid growth after more than 90 passages in culture that is shared with most brain cell lines (Fu et al., 2015; Hasoon

et al., 2011; Zheng et al., 2015) but not with the sea bass SSB-W1 long-term which show slower kinetics (after 24 passage could be considered a continuous line) (Servili et al., 2009). In addition, DLB-1 cells are not sensitive to freeze-thawing cycles and are capable of transfection but with low efficacy, as it occur with the other evaluated fish brain cell lines. In general, fish cell lines show lower transfection efficiency than human or murine cell lines but are also useful to evaluate protein function.

Upon morphological characteristics, DLB-1 cell cultures showed mixed cell morphologies with most of the cells being epithelioid-like cells and some star-like cells resembling astrocytes. In the literature, different morphologies have been described for fish brain cell lines including fibroblastic (Chen et al., 2010; Fu et al., 2015; Hasoon et al., 2011; Lai et al., 2001a) epithelial (Ku et al., 2009; Servili et al., 2009; Wang et al., 2010; Wen et al., 2008a, b; Zheng et al., 2015), endothelial (Bloch et al., 2015) or neuronal (Hinsch and Zupanc, 2006; Wen et al., 2010). Because the morphological features of cell lines are not easily related with its origin some studies have attempted to characterize them using markers for the main brain cells, neurons or glial cells. In the case of DLB-1 cells our data firmly suggest that they belong to the glial linage since they transcribe *gfap* and *coro1a* and not *rbfox3* and *map2* (neuronal markers). However, data are not very conclusive in most of the related fish cell lines. For example, some authors have described the presence of glial fibrillary acidic protein (GFAP) but lacked to evaluate the expression of other neural markers (Wang et al., 2015; Zheng et al., 2015). Interestingly, in the previous European sea bass brain long-term cultures, SSB-W1 cells, the characterization was more complete and found expression of nestin, GFAP and neurofilament (NF) proteins, markers for both glial and neural cells, suggesting that these cells could be neural stem cells (Servili et al., 2009) as well as other authors found that fish neural-like stem cells were positive for GFAP immunostaining (Wen et al., 2008b). Thus, our data about DLB-1 cell morphology and gene expression points to the need to further analysis. More efforts should be made to characterize the generated DLB-1 cell line and seek for its usefulness in studies concerning nervous system and neurobiology.

After a partial characterization of the DLB-1 cells we tested whether they could be used for toxicological purposes and evaluated the cytotoxic effects of several metals. Though many toxicological studies have used fish cell lines (Bols et al., 2005), brain cell lines

have just been used for virological studies (Lakra et al., 2011) and only one has found that eelB cells failed to respond to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Bloch et al., 2015). Our data show that DLB-1 cells exposed to metals produce a dose-dependent cytotoxic effect. Some differences were observed depending on the detection method used (MTT or NR) but in both cases the most and less toxic metals were Cd and Pb, respectively. These differences could reside in the cytotoxic mechanism used by metals and in the detection method. Thus, data suggest that Cd and MeHg alter lysosomes earlier or to a greater extent than As and Pb as indicated by NR method while mitochondria are more affected by Cd and As. According to the MTT assay, the metal toxicity to DLB-1 cells is in the order of Cd>As>MeHg>>>Pb while in the gilthead seabream SAF-1 and grass carp ZC-7901 cell lines the order was MeHg>As>Cd>Pb (Morcillo et al., 2016) and Cd>Hg>Pb>Cu>Cr>As (Xiang et al., 2001), respectively. However, this is the first study in which metal cytotoxicity has been evaluated in a fish brain cell line. In fact, neurotoxicologial effects of Pb and MeHg are probably the best characterized but other metals such as Cd, As, Mn, Fe, Zn or Cu have also great impact on the nervous system in mammalian species (Wright and Baccarelli, 2011) and also in fish (Ceccatelli et al., 2010; Kennedy, 2011). Interestingly, Hg is one of the most neurotoxic metals but this was not the case for the DLB-1 cells and which are the mechanisms involved in this cytotoxicity should be further studied. It is known that one of the first consequences of metal poisoning is the increased production of ROS. DLB-1 cells exposed to Cd and Pb showed a great production of ROS while MeHg and As failed to do so in contrast to what has been reported to other fish cell lines in which most metals induced ROS production (Ariza et al., 1998; Fernández et al., 2013; Morcillo et al., 2016; Romero et al., 2003; Sandrini et al., 2009). However, the ROS production induced by Cd and Pb did not correlated with DLB-1 cells mortality pointing these data to the involvement of other mechanisms, as previously found and suggested in the SAF-1 cell line (Morcillo et al., 2016). Strikingly, all the metals produced a similar decrease in the antioxidant system as determined by the transcriptional down-regulation of *cat* and points to an increased ROS production. Interestingly, MeHg and As exposure up-regulated the transcription of *mt* in DLB-1 cells, which is one of the most important protective proteins against metal poisoning. In fact, MTs have been proposed as important ROS scavengers (Ruttkay-Nedecky et al., 2013). Therefore, this enhanced *mt* gene expression explained the low levels of ROS

production induced by MeHg and As in the DLB-1 cells. A similar pattern was also evidenced in the seabream fin SAF-1 cells in which the highest *mt* transcription is correlated with the lowest ROS production pointing to their importance in cellular protection and as ROS scavengers. Unfortunately, none of these aspects have been evaluated in other available fish brain cell lines until now. Metals exposure produce alterations in the proliferation of neural cells and induces cell apoptosis (Ceccatelli et al., 2010; Corniola et al., 2008; Matés et al., 2010). Our data also demonstrate that DLB-1 exposure to the EC_{50} of metals provokes a decrease in the cell mitogenesis that is more evident in the case of Pb and As that completely abrogated the G2/M phase of the cell cycle. Similarly, it has been described that metal exposure to brain cells resulted in decreased cell proliferation by G2/M arrest accompanied with decrease in cyclin B1 and increased p53 expression (Ponce et al., 1994; Zhao et al., 2002). Finally, overproduction of ROS together with the expression of other proteins such as p53, led to cells to cell death, usually apoptosis (Rana, 2008). Our data showed that DLB-1 cells exposed to EC_{50} of metals also died by apoptosis as demonstrated by the increase of sub-G0/G1 cells, annexin staining and down-regulation of the anti-apoptotic *bcl2* expression. Interestingly, no necrosis cell death was detected indicating that only apoptosis cell death is occurring in the exposed DLB-1 cells. In contrast to our data, in fish cell lines and primary cell cultures exposed to metals the cell death is mainly by apoptosis but also by necrosis and/or necroptosis (Krumschnabel et al., 2005; Morcillo et al., 2015a, b, 2016; Selvaraj et al., 2013).

Chapter 2

Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line

Abstract

The use of cell lines to test the toxicity of aquatic pollutants is a valuable alternative to fish bioassays. In this study, fibroblast SAF-1 cells from the marine gilthead seabream (*S. aurata*) were exposed for 24 h to the metals Cd, Hg, MeHg, As or Pb, and the resulting cytotoxicity was assessed. NR, MTT, CV and LDH viability tests showed that SAF-1 cells exposed to the above metals produced a dose-dependent reduction in the number of viable cells. MeHg showed the highest toxicity (EC_{50} = 0.01 mM), followed by As, Cd, Hg and Pb. NR was the most sensitive method followed by MTT, CV and LDH. SAF-1 cells incubated with each of the metals also exhibited an increase in the ROS species and apoptosis cell death. Moreover, the corresponding gene expression profiles pointed to the induction of the MTs protective system, cellular and oxidative stress and apoptosis after metal exposure for 24 h. This report describes and compares tools for evaluating the potential effects of marine contamination using the SAF-1 cell line.

1. Introduction

Industrial and mining activities often lead to a significant local increase in the levels of chemical pollutants, such as metals (Sapkota et al., 2008), which can seriously affect aquatic communities (Uysal et al., 2009). Metals are not biologically or chemically degraded but may be bioaccumulated in the food chain since they are found at high concentrations in fish living in contaminated waters (Gibbs and Miskiewicz, 1995; Tariq et al., 1993). This is also of relevance in the case of cultured fish which are fed diets containing fish oils and meals. The accumulation of toxicants may have a negative impact not only for the fish but also for consumers of the fish (Fernandes et al., 2008). In fact, fish are known to be the greatest source of toxic trace elements (Hg, Se, Cd, Pb, As, Cu, Cr, Fe, Mn, Mb, Va and Zn) in humans. Thus, how metals are accumulated and how they can affect cultured fish as well as the levels they transfer to humans is important information for the aquaculture industry to develop.

In fish, as in mammals, the use of cell lines has been successfully applied for toxicological purposes by measuring viability, morphology, metabolism, cell attachment/detachment, cell membrane permeability, proliferation or growth kinetics (Bols et al., 2005; Fent, 2001). Indeed, many cytotoxicity studies have positively related the *in vitro* and *in vivo* EC_{50} values in fish exposed to different contaminants, demonstrating the value of using fish cell lines in this field of research (Castaño et al., 2003; Fent, 2001; Segner, 2004; Vega el al., 1996). Most of the fish cell lines (RTG-2, BF-2, CHSE-124, EPC and, more recently, zebrafish-derived cell lines) used in aquatic toxicology are derived from freshwater species including salmonids or cyprinids. Unfortunately, there are very few deposited marine fish cell lines available to screen and evaluate the toxicology in marine fish species *in vitro*. Thus, further research into the effects of metals on cultured fish species and their cell lines, including marine fish, should be undertaken, not only from an environmental point of view but also bearing in mind the interests of the aquaculture industry and consumers.

Although some studies have evaluated certain aspects of gilthead seabream (*S. aurata)* biology after exposure to metals *in vivo* (García-Santos et al., 2011; Guardiola et al., 2013a, b; Souid et al., 2013), to our knowledge, only one has evaluated the *in vitro* toxicity of Cd and Cu on the SAF-1 cell line, derived from the fin of seabream

(Minghetti et al., 2011). Their results showed that Cd is more cytotoxic than Cu to SAF-1 cells after 24 h of exposure and the microarray study found the implication of Cu transporters (CTR1, ATP7A and ATOX1), MT and oxidative stress (GR and Cu/Zn SOD) genes in response to Cu exposure. Thus, the aim of the present study was to assess the cytotoxicity effects of four metals (Cd, Hg, As and Pb) on the SAF-1 cell line from gilthead seabream, one of the most widely cultured and consumed fish in the Mediterranean area. For this, cell viability, the production of ROS, the expression of genes related to cell protection or oxidative stress and the cell-death mechanism were evaluated.

2. Materials and methods

2.1. SAF-1 cell culture

The established cell line SAF-1 (ECACC 00122301, Public Health England) (Béjar et al., 1997) was seeded in 75-cm^2 plastic tissue culture flasks (Nunc, Denmark) and cultured at 25ºC in an incubator with an atmosphere with 85% relative humidity using L-15 Leibowitz medium (Life Technologies, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 µg/mL streptomycin (Life Technologies) and 100 U/mL penicillin (Life Technologies). The subculture was done every 4-5 days with 0.25% (w/v) trypsin (Sigma) plus 0.02% (w/v) etylendiaminotetracetic acid (EDTA) solution (Sigma-Aldrich, Madrid, Spain).

2.2. Metal exposure

Different salts of the metals (Sigma-Aldrich) were used: Cd chloride ($CdCl_2$), MeHg (II) chloride [CH_3HgCl (MeHg)], mercury (II) chloride ($HgCl_2$), lead (II) nitrate ($Pb(NO_3)_2$) and trioxide As (As_2O_3).

SAF-1 cells were plated in 96-well plates at 2.5×10^4 cells/well, cultured overnight and washed with fresh medium. Then, 180 µL/well of fresh culture medium plus 20 µL/well of water (controls) or metal were daily prepared to make the following final concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 mM of Cd or As; 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 and 0.1 of MeHg; 0.001, 0.05, 0.01, 0.1, 0.25, 0.4 and 1 mM of Hg; or 1, 2, 3, 4 or 5 mM of Pb. Cells were exposed for 24 h at 25ºC in the incubator.

The cytotoxicity was determined in three independent experimental tests and each test was performed with seven wells for each of the metals or controls.

2.3. Cytotoxicity assays

2.3.1. Neutral Red

Neutral red (NR) uptake was used to assess cell viability (Borenfreund and Puerner, 1985). After 24 h of exposure, cells were washed with 10 mM phosphate saline buffer (PBS) and 100 µL/well of 0.33% neutral red solution (Sigma) added to the SAF-1 cell cultures. After incubation (3 h at 25ºC) the cells were washed and fixed with 1% acetic acid and 50% absolute ethanol and the absorbance was measured at 540 nm and 690 nm in a microplate reader (BMG Labtech, Madrid, Spain). Blanks consisted of wells without cells.

2.3.2. MTT

The MTT test is based on the reduction of the yellow soluble tetrazolium salt (MTT) into a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase (Mosmann, 1983). After exposure, cells were washed and 200 µL/well of culture medium containing 1 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma). After 4 h of incubation at 25ºC, cells were washed, formazan solubilized and the absorbance at 570 nm and 690 nm was determined. Blanks consisted of wells without cells.

2.3.3. Crystal violet

Crystal violet (CV) staining is usually related to the number of viable cells since dead cells are detached and washed out (Gillies et al., 1986). After exposure, cells were washed and fixed with 100 µL/well of Carnoy (3:1 v/v Methanol/Acetic acid; Sigma) for 15 min. Then, cells were stained with 0.1% crystal violet (N-hexamethylpararosaniline; Sigma) for 30 min, washed and solubilized with 10% methanol and 5% acetic acid solution. The plates were read at 570 nm and 595 nm. Blanks consisted of wells without cells.

2.3.4. Lactate dehydrogenase (LDH) assay

Cytotoxicity induced by metals was assessed by lactate dehydrogenase (LDH) leakage into the culture medium (Korzeniewski and Callewaert, 1983). Following exposure, the culture medium was recovered and centrifuged at 250 g for 4 min. The activity of LDH in the supernatant was measured using the commercial kit CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madrid, Spain). Briefly, 50 µL of supernatant was incubated with 50 µL of CytoTox 96® Reagent for 30 min at room temperature, stopped with 25 µL of Stop Solution and the absorbance read at 490 nm. Samples of unexposed cells were completely lysed by the addition of 2 µL of Lysis Solution for 45 minutes and the absorbance was related to the 100 % of cell lysis. Blanks consisted of wells without cells.

2.3.5. Data analysis

Experiments were performed thrice with seven replicates for each exposure concentration. The viability in each sample was determined according to the controls or unexposed cells (100% viability). Intra- and inter-assay coefficient of variation was lower than 8.5 and 12.3 %, respectively. For each method, cell viability data and the logarithm of metal concentrations were represented and fitted with an exponential decay 3 parameter curve [$f = y0 + a \times \exp(-bx)$]. Fitted curves always showed r^2 values higher than 0.92. The concentration producing 50% cell death (EC_{50}) was determined for all metals and assays using Sigma plot software while EC_{10} value was only obtained in some cases. The EC_{50} used in other assays were calculated with the data obtained by using the NR method whilst the EC_0 dosages were referred to the lowest concentration assayed (producing a nonsignificant cytotoxicity).

2.3.6. Light microscopy

After application of the NR, MTT and CV methods some samples were examined under a microscope (Nikon Eclipse TE 2000-U; Nikon, Madrid, Spain) and photographs were taken by a digital camera (Nikon DS-5M; Nikon).

2.4. ROS production

Dihydrorhodamine 123 (DHR; Life Technologies) was used to measure ROS production (Henderson and Chappell, 1993). DHR 123 is able to diffuse cell membranes and when oxidized by ROS (mainly by hydrogen peroxide) becomes green fluorescent rhodamine 123 which is sequestered into the mitochondria. After 24 h of exposure to the EC_0 and EC_{50} dosages of each metal, SAF-1 cells were detached by trypsin/EDTA solution, resuspended in 200 µL of fresh medium with 5 µM of DHR 123 and incubated for 30 min at 25ºC. The samples were then analyzed in a flow cytometer (Becton Dickinson, Madrid, Spain) to ascertain the extent of ROS production as indicated by the mean green fluorescence intensity for each treatment.

2.5. Expression of genes

After exposure to EC_0 and EC_{50} of metals the supernatant was aspirated and TRIzol Reagent (Life Technologies) added to the wells in order to extract the total RNA. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 mg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT_{18} primer. The expression of the selected genes was analysed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Life Technologies) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 µL of 2 × SYBR Green supermix, 5 µL of primers (0.6 mM each) and 5 µL of cDNA template) were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample. Gene names follow the accepted nomenclature for zebrafish (http://zfin.org/). Genes and primers are shown in Table 3. In all cases, each PCR was performed with triplicate samples.

2.6. Scanning electron microscopy (SEM) analysis of cell morphology and apoptosis

Sterile glass cover slips were placed in 6-well cell culture plates (Nunc) and 7.5×10^4 SAF-1 cells/well were cultured overnight. Cells were then exposed to the EC_{50} of each metal for 24 h. The cover slips were washed, fixed in 3% (v/v) glutaraldehyde and post-

fixed in 1% OsO_4. Samples were dehydrated in acetone, critical-point dried, sputter coated with gold and examined with a Jeol 6.100 scanning electron microscope.

2.7. Statistical analysis

Data of the cytotoxic effects are presented for each metal and colorimetric method using the fitted curves. The results of ROS production are expressed as mean ± standard error, SE. Gene expression is expressed as relative expression to the house-keeping gene. Data were statistically analyzed by two-way analysis of variance (ANOVA; $P \leq 0.05$) to determine differences among metal concentration as well as among metals, followed by a post-hoc Tukey test.

Table 3. Oligonucleotide primers used for real-time PCR

Group	Gene	Abbreviation	Acc. number	Primer sequence (5´→3´)
House-keeping	Elongation factor 1-alpha	*ef1a*	AF184170	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG
Cellular protection	Metallothionein-A	*mta*	X97276	F: ACAAACTGCTCCTGCACCTC R:CAGCTAGTGTCGCACGTCTT
Cellular stress	Heat-shock protein-70	*hsp70*	EU805481	F: AATGTTCTGCGCATCATCAA R: GCCTCCACCAAGATCAAAGA
Oxidative stress	Catalase	*cat*	FG264808	F: TTCCCGTCCTTCATTCACTC R: CTCCAGAAGTCCCACACCAT
	Cu/Zn superoxide dismutase	*sod*	AJ937872	F: CCATGGTAAGAATCATGGCGG R: CGTGGATCACCATGGTTCTG
	Glutathione reductase	*gr*	AJ937873	F: CAAAGCGCAGTGTGATTGTGG R: CCACTCCGGAGTTTTGCATTTC
	Peroxiredoxin 1	*prx1*	GQ252679	F:CTCCAAGCAATAATAAGCCCAAAG R:TCACTCTACAGACAACAGAACAC
	Peroxiredoxin 2	*prx2*	GQ252680	F: CAAGCAGTAAATGTGAAGGTC R: GATTGGACGCCATGAGATAC
Apoptosis	B-cell lymphoma 2	*bcl2*	FM145663	F: CAGCCAGGTGCTGACATAGA R:TCAGGAGTGATGTCGAGCTG
	Bcl-2 associated X protein	*bax*	AM963390	F:CAACAAGATGGCATCACACC R:TGAACCCGCTCGTATATGAAA

3. Results

3.1. Cytotoxicity assays

SAF-1 cells exposed to metals showed cytotoxicity as seen by light microscopy and colorimetric assays (Figure 16). Images of NR, MTT or CV indicate a decrease in the number of adherent cells after 24 h exposure to metals (Figure 16A-F). The absorbance assays show dose-dependent cytotoxicity from which reliable EC_{50} values could be calculated (Figure 16; Table 4) whilst EC_{10} values could only be determined for all the metals in the NR assay. MeHg was generally the most toxic followed by As, Cd, Hg and

Pb (Table 4). Among the colorimetric methods, NR uptake was the most sensitive, (except for As), followed by the MTT, CV and LDH assays. Thus, NR method was the only one giving a 100% of cell death for all the metals (Figure 16), whilst the other methods never reached this percentage for all the treatments, what allowed us to calculate all the EC_{10} values in the NR assay but not all in the others.

Table 4. Values of r^2, EC_{10} and EC_{50} (mM) after exposure of SAF-1 cells to metals for 24 h. NR, neutral red, MTT, yellow soluble tetrazolium salt; CV, crystal violet; LDH, lactate dehydrogenase; ND, not determined.

Heavy metals	NR			MTT			CV			LDH		
	r^2	EC_{50}	EC_{10}	r^2	EC_{50}	EC_{10}	r^2	EC_{50}	EC_{10}	r^2	EC_{50}	EC_{10}
Cd	0.96	0.1	0.37	0.98	0.1	0.32	0.99	0.160	0.52	0.98	0.703	ND
MeHg	0.97	0.015	0.049	0.96	0.018	0.055	0.99	0.021	0.07	0.97	0.018	0.047
Hg	0.96	0.160	0.5	0.97	0.3	ND	0.95	0.3	ND	0.99	0.363	ND
Pb	0.99	3.2	3.98	0.96	3.4	ND	0.96	4.5	ND	0.98	4.2	ND
As	0.99	0.082	0.28	0.92	0.03	0.14	0.99	0.367	0.85	0.94	0.226	0.5

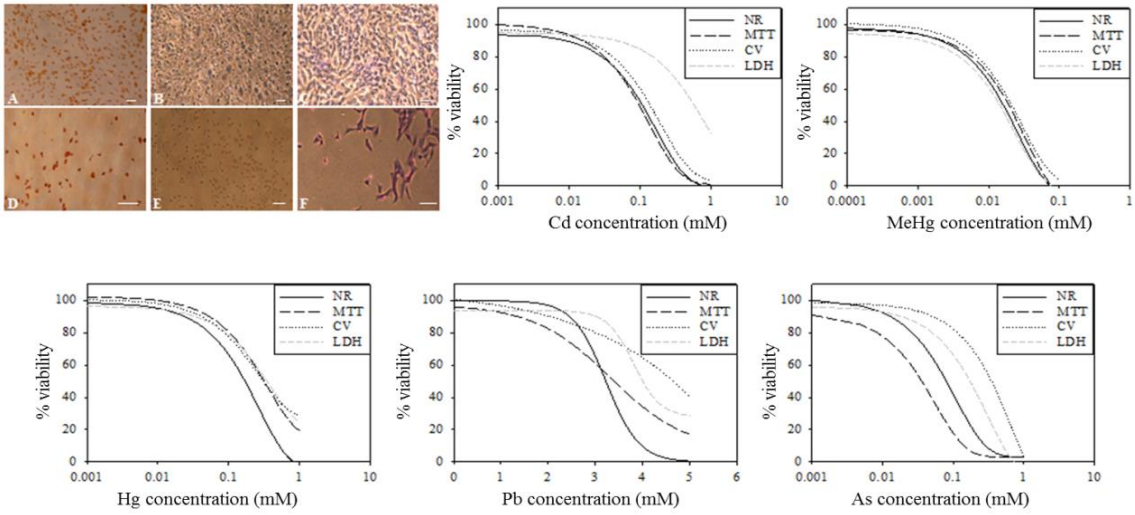

Figure 16. Representative light microscopy images and cytotoxicity curves of SAF-1 cells exposed to different metals for 24 h. SAF-1 cells unexposed (controls; A-C) or exposed to different metals for 24 h (D, 0.1 mM Hg; E, 0.5 mM Cd; F, 0.1 mM As) and stained with NR (A, D), MTT (B, E) or CV (C, F). Scale bar is 100 mm. In the cytotoxicity curves, lines represent the fitted curve for each method: NR, neutral red, MTT, yellow soluble tetrazolium salt; CV, crystal violet; LDH, lactate dehydrogenase.

3.2. Metals induce the production of ROS

SAF-1 cells exposed to the EC_0 and EC_{50} of all the metals for 24 h showed a significant increase in the production of ROS compared to the controls ($P<0.05$) (Figure 17). ROS production by SAF-1 cells exposure to the EC_{50} of MeHg, Hg and As produced significantly higher levels than in those exposed to EC_0, especially in the case of As, whilst Cd and Pb induced the same ROS production at both. Moreover, when the metals were compared, As induced the greatest effects followed by mercurial compounds, Pb and Cd.

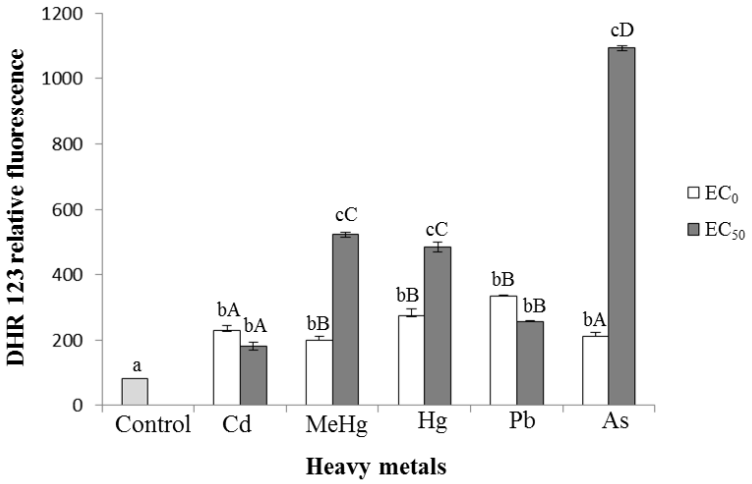

Figure 17. Production of ROS, expressed as relative fluorescence of DHR 123, by SAF-1 cells after exposure to EC_0 and EC_{50} of metals for 24 h. Bars represent the mean ± SEM (n = 3 separate experiments). Statistically significant differences ($P<0.05$) were studied by two-way ANOVA. Different lower case letters indicate significant differences among dosages (control, EC_0 and EC_{50}) whilst different capital letters denote significant differences among the metals.

3.3. Gene expression profiles indicate protection, oxidative stress and apoptosis

Exposure of SAF-1 cells to the EC_0 and EC_{50} of the metals produced regulation in the expression of genes related to metal protection, cellular and oxidative stress, as well as apoptosis (Figure 18). Firstly, we evaluated the cellular protection to metals by the expression of the *mta*. As expected, Cd, MeHg, Hg and Pb significantly up-regulated *mta* transcription in SAF-1 cells whilst As exposure produced a down-regulation when compared with the controls. Strikingly, the EC_0 produced greater up-regulation than the EC_{50} of Cd and Hg while in MeHg exposed cells the opposite occurred. In addition,

very clear significant differences were observed between As and the other metals (Figure 18) when they were compared.

Regarding cellular and oxidative stress gene expression, SAF-1 cells exposed to Cd failed to alter the expression of *hsp70*, *prx1* and *prx2*, *sod* and *gr* genes but up-regulated *cat* transcription when compared with the controls (Figure 18). By contrast, MeHg exposure produced a significant increase in the expression of all these genes, up-regulation that was usually higher when SAF-1 cells were incubated with the EC_{50} compared with the unexposed cells. However, Hg produced no significant effect (*hsp70*), up-regulation (*sod*, *cat* or *gr*) or down-regulation (*prx1* and *prx2*) in the gene transcriptions and the effects were much higher with the EC_{50} than with the EC_0. Pb, by its own, was mainly inducing an up-regulation of all these genes. Finally, As exposure produced in the SAF-1 cells a significant down regulation in the transcription

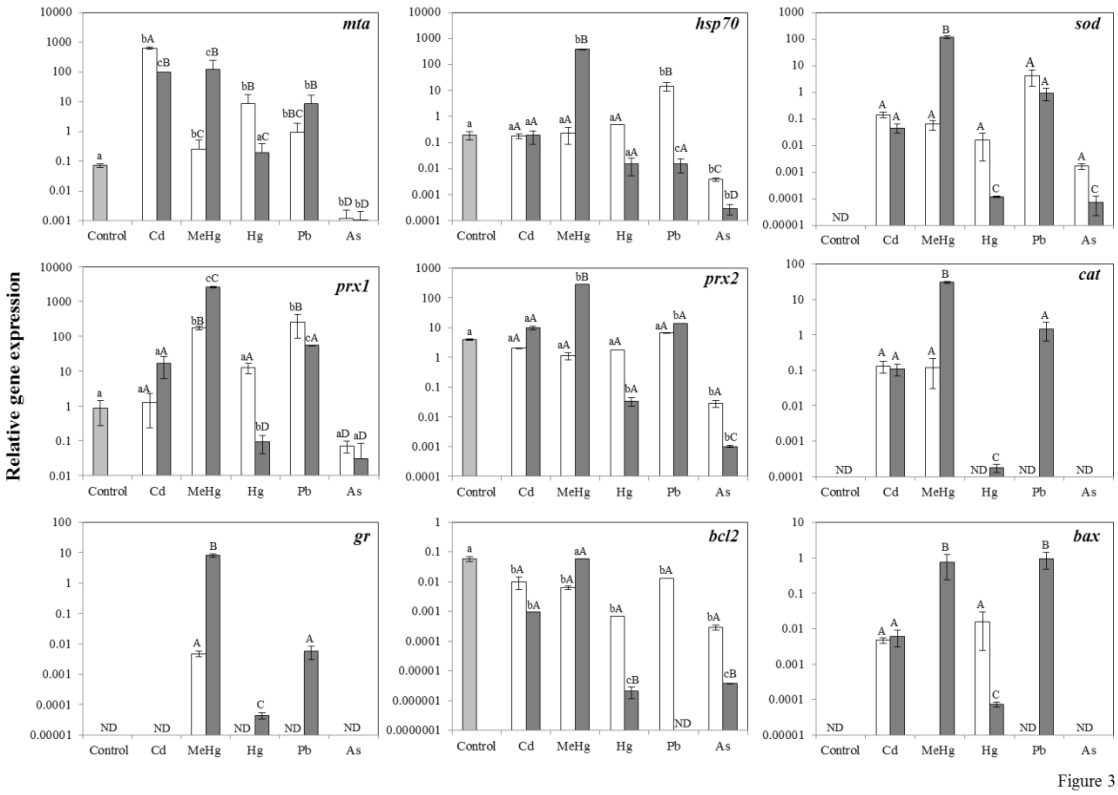

Figure 18. Gene expression of SAF-1 cells unexposed (light gray) or treated with EC_0 (white bars) and EC_{50} (dark grey bars) of metals for 24 h. Bars represent the mean ± SEM (n = 3 separate experiments) gene expression relative to the expression of the endogenous control *ef1a* gene. Statistically significant differences ($P<0.05$) were studied by two-way ANOVA. Different

lower case letters indicate significant differences among dosages (control, EC_0 and EC_{50}) whilst different capital letters denote significant differences among the metals. ND, not detected.

of *hsp70*, *prx1* and *prx2*, up-regulation of *sod* while the expression of *cat* and *gr* genes was undetected, as in the controls. When the different metals were compared, Cd, Hg and Pb produced quite similar effects being MeHg and As the most different ones.

The expression on the anti-apoptotic B-cell lymphoma-2 gene (*bcl2*) and the pro-apoptotic Bcl2-associated X gene (*bax*) was also evaluated (Figure 18). Thus, SAF-1 cells exposed to metals showed a significant down-regulation of *bcl2* transcription in all cases, except in those exposed to the EC_{50} of MeHg, when compared with the control cells. Moreover, when the metals were compared, the EC_{50} of Hg, Pb and As produced a much higher reduction in the *bcl2* transcription than Cd and MeHg. Finally, *bax* transcription was undetected in control cells and exposed to As whilst it was greatly expressed in Cd, MeHg, Hg and Pb exposed cells suggesting the cell death by apoptosis. In addition, the induction provoked by MeHg and Pb was significantly higher than the effects produced by the other metals.

3.4. Cell morphology showed apoptotic features

SEM images showed the control cells to have wide and short cytoplasmic extensions and intact cell surfaces (Figures 19A, 19B). After metal exposure, the number of cells was greatly reduced (Figure 19C) and those remaining presented modified extensions in the width, length and number (Figures 19D-G). The most prominent characteristic of the treated cells was the abundance of membrane blebs resembling those produced in apoptosis cell-death, indicating that this is the induced dying mechanism (Figures 19E, 19F). Finally, some cells showed holes in the membrane or even lost their membrane, indicating that they were in very late apoptosis or necrosis (Figures 19G, 19H).

4. Discussion

In vitro cytotoxicity assays with fish cell lines represent technically easy to handle and reproducible test systems that provide very good correlation with acute lethality tests with fish *in vivo* (Segner, 2004). Unfortunately, most of the information focusing on fish

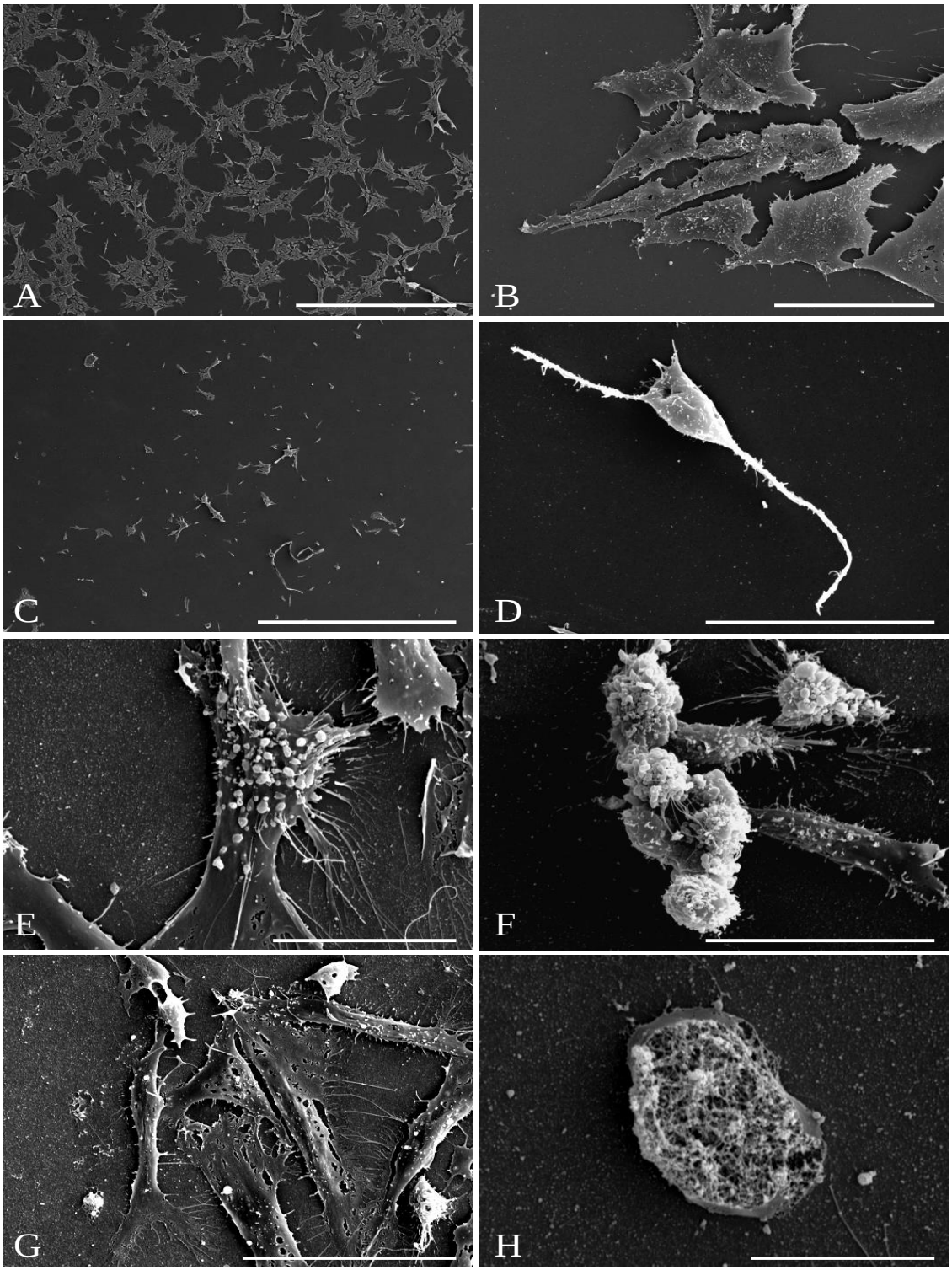

Figure 19. Representative scanning electron microscopy images of SAF-1 cells unexposed (control; A, B) or exposed to EC_{50} of As (C), Cd (D), Pb (E), Pb (F) or MeHg (G, H). Scale bars: 1 mm (A, C); 50 μm (B); 30 μm (D, F); 20 μm (E, H); 60 μm (G).

toxicology comes from freshwater species and less is known about marine fish species and their cell lines. This is especially true for the gilthead seabream, the most important marine cultured species in the Mediterranean Sea. Therefore, the cytotoxicity of Cd, MeHg, Hg, Pb and As on the seabream SAF-1 cell line, as well as morphological alterations and changes in ROS production and in the regulation of different genes (related to cellular protection, oxidative stress and apoptosis) were analysed in order to increase our knowledge of metal toxicity and the mechanisms involved in marine fish cell lines.

4.1. Cytotoxicity

Among the cytotoxicity tests used *in vitro*, colorimetric NR, MTT, LDH release and CV methods were used to evaluate lysosomal damage, mitochondrial impairment, membrane integrity and cell attachment/detachment, respectively. The cytotoxicity assays used revealed different profiles, with NR and the MTT assays being the most sensitive, whereas the CV and LDH methods were only suitable after exposure to high metal concentrations or long time. This is supported by several authors (Minghetti et al., 2011; Segner, 1998), who found that the EC_{50} are usually higher for LDH leakage assay and protein assays (like the CV method) than for NR and MTT assays. This demonstrates that organelle impairment occurs before any permanent cell membrane damage and cell detachment occurred. Similarly to our results, in the SAF-1 cell line, the NR assay showed higher sensitivity than the kenacid blue method (Minghetti et al., 2011), which is based on protein determination, and similar to the CV assay. For Cd, MeHg and Pb, the NR and MTT curves were almost identical and gave very similar EC_{50} values, suggesting that both lysosomes and mitochondrial organelles are impaired at similar metal concentrations (Cornelis et al., 1992; Maracine and Segner, 1998; Vian et al., 1995). Moreover, EC_{50} data suggest that lysosomes are the most affected organelles by Hg and mitochondria by As.

Our observations show that NR is the most sensitive technique and the only one in which the 100% of cell death was always observed. Thus, the NR EC_{10} and EC_{50} values indicated that MeHg was the most toxic metal for the SAF-1 cells followed by As, Cd, Hg and Pb, which is consistent with previous literature. For example, in the freshwater rainbow trout (*Oncorhynchus mykiss*) fish cell line RTG-2, the most toxic metal was Hg, followed by Cd, Zn, Cu, Pb and Ni (Maracine and Segner, 1998) whilst in the case

of the grass carp (*Ctenopharyngodon idella*) ZC-7901 cells the order was $Cd^{2+}>Hg^{2+}>Pb^{2+}>Cu^{2+}>Cr^{6+}>As^{5+}$ (Xiang et al., 2001). In a previous paper, the SAF-1 cells were more sensitive to Cd compared to Cu, and the EC_{50} values for Cd (Minghetti et al., 2011) were very similar to our results. However, rainbow trout RTG-2 or bluegill (*Lepomis macrochirus*) BF-2 cells exposed to Hg show EC_{50} values of 0.012 and 0.019 mM (Bayoumi et al., 1999), respectively, which are about ten-fold lower than in the SAF-1 cell line (EC_{50}=0.16 mM). A similar pattern was observed when the RTG-2 and BF-2 cells were incubated with Cd or Pb (Bayoumi et al., 1999) indicating that SAF-1 cells are more resistant to metals. This kind of differences in the cell lines susceptibility could be due to the source of the cell lines (fish species or tissue), incubation temperature, metal form, solubility, interactions with culture medium components, etc. In example, RTG-2 cells are more resistant to Hg, Cd, Cu or Ni when they are incubated in presence of 10% FBS than with 0% FBS (Segner, 1998) while they are also more tolerant to metals than BF-2 cells (Bayoumi et al., 1999).

4.2. Cellular oxidative stress

SAF-1 cells exposed to all the metals also showed a significant increase in the cellular ROS levels, as indicated by DHR 123 fluorescence (Figure 17). ROS production has been also demonstrated in fish cell lines exposed to Pb, Hg, As, Cu or Zn (Ariza et al., 1998; Fernández et al., 2013; Romero et al., 2003; Sandrini et al., 2009; Selvaraj et al., 2013) and it is widely accepted that these ROS are the main inducers of the cytotoxicity (Benedetti et al., 2007; Loro et al., 2012; Monteiro et al., 2010; Wang et al., 1996). However, our data are not fully supporting this hypothesis. While the ROS production followed the order As>MeHg>Hg>Pb>Cd the cytotoxic effect was MeHg>As>Cd>Hg>Pb. Thus, SAF-1 cells exposed for 24 h to Cd and Pb produced the lowest ROS levels and not followed a dose-response suggesting that these radicals are not the most important inducers of the cytotoxicity mediated by Cd and Pb. By contrast, MeHg, Hg and As induced ROS production in a dose-dependent manner pointing to the importance of ROS in the cell death. Interestingly, the EC_{50} of As produced the highest ROS level in the SAF-1 cells, which agrees with studies in mammalian (Wang et al., 1996) and fish (Selvaraj et al., 2013; Wang et al., 2004b) cells exposed to As. Moreover, it has been shown that the oxidation of As under physiological conditions results in H_2O_2 formation (Valko et al., 2005), what could partly explain the very high ROS levels

in our assays. In addition, As exposure of two fish cell lines resulted in apoptosis cell death mediated by ROS (Wang et al., 2004b) supporting our observations for As, and probably for MeH and Hg, in the seabream SAF-1 cell line.

This generation of hydrogen peroxide is mainly produced at mitochondrial level by SOD while its elimination may involve CAT, GR and PRXs systems (Di Giulio and Meyer, 2008; Lushchak, 2011). Our results show that the moderate ROS production after SAF-1 cells exposure to Cd, MeHg, Hg or Pb metals is concomitant with the increased *sod* gene transcription and the scavengers *cat*, *gr*, *prx1* and *prx2*. In fact, MeHg was the metal inducing the highest antioxidant response suggesting the scavenging and clearance of the ROS. However, As induced the highest ROS production and the lowest *sod* transcription supporting the idea that part of the ROS come from the direct As oxidation above mentioned. In addition, As failed to induce any of the antioxidant genes, making the SAF-1 cells unable to clear the ROS, suggesting different toxicological mechanisms for the As compounds. In SAF-1 cells, *sod* expression was unaltered after exposure to Cu, Zn or Cd for 24 h but down-regulated after 4 h (Minghetti et al., 2011) though they did not measure ROS production. However, this gene was down-regulated in zebrafish (*Danio rerio*) embryos exposed to Cd (Hsu et al., 2013) or crucian carp (*Carassius auratus* var. *Pengze*) exposed to Cr (Li et al., 2013) even when the ROS were overproduced indicating other sources for ROS generation apart from mitochondrial SOD enzyme. At protein level, As exposure has been seen to induce (Bhattacharya and Bhattacharya, 2007) or decrease (Altikat et al., 2014) SOD activity in fish. Interestingly, As induced the CAT activity in the cytosol but decreased that of the peroxisome and this has been related with increased cellular ROS and As toxicity (Bhattacharya and Bhattacharya, 2007). SOD, CAT and glutathione system have shown different patterns and some controversy in fish exposed to metals, but still considered good biomarkers of the oxidative stress produced by metals (Di Giulio and Meyer, 2008), though the role of the PRXs system has never been evaluated in fish exposed to metals. In fact, *prx2* has been demonstrated to play a role as antioxidant by protecting a human cell line against oxidative damage by hydrogen peroxide (Kim et al., 1997) and could also be involved in removing the ROS induced by metals. Therefore, the relation between ROS production and oxidative/antioxidant response should be further investigated in fish.

4.3. Cellular protection and stress

MTs are among the most important proteins in protecting cells against metal toxicity, especially Cd, Hg, Cu and Zn. Our results show that the *mta* gene is up-regulated by Cd, MeHg, Hg and Pb in SAF-1 cells, as previously documented in fish (Bourdineaud et al., 2006; Minghetti et al., 2011). In addition, MT has been shown to directly react with hydroxyl radicals in cell-free *in vitro* systems (Thornalley and Vasak, 1985) and may, consequently, act as a donor of reduced thiolate groups, thereby fulfilling a role in protecting the cell from free radical mediated injury and reducing oxidative stress (Lazo et al., 1995). Surprisingly, As produced a significant down-regulation of the *mta* expression as well as the highest ROS production, pointing to the importance of MT protein in ROS scavenging in SAF-1 cells as previously suggested (Ruttkay-Nedecky et al., 2013). Similarly to *mta*, a cell stress biomarker, the heat-shock protein 70 (*hsp70*) gene followed a quite parallel pattern demonstrating cell injury, whilst As produced again a significant down-regulation. In seabream, *in vivo* exposure to Cd produced up-regulation of *mt* gene and down-regulation of *hsp70* (Sassi et al., 2013), whilst at protein level the MT was unaffected and the HSP70 increased (García-Santos et al., 2011).

4.4. Cell death

Finally, a well-known consequence of imbalanced oxidative stress is cell death by apoptosis. In the exposed SAF-1 cells we identified morphological changes characteristic of the apoptosis process although the necrosis pathway cannot be completely excluded. Moreover, the expression of apoptosis regulatory genes confirms this finding. Thus, pro-apoptotic *bax* gene was up-regulated, except by As, and the anti-apoptotic *bcl2* down-regulated by metals confirming this cell death pathway. However, control of apoptosis is regulated by balance of pro and anti-apoptotic proteins and many other regulatory factors. Strikingly, in the case of As, SAF-1 cells suffered apoptosis cell death accompanied by down-regulation of *bcl2* transcription, whilst *bax* was not detected, what suggests that this regulation occurred at either protein level or implicated other pro-apoptotic regulators. Moreover, in the case of MeHg, several studies have confirmed that the fish cell death after metal exposure is mainly by apoptosis (Luzio et al., 2013; Risso-De Faverney et al., 2004). However, few papers have dealt with this aspect in fish cell lines. For example, as in our study, nuclear condensation, holes in the

membrane and distinct apoptotic-like bodies were observed in the grass carp ZC-7901 cell line after exposure to Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}, As^{5+} and Pb^{2+}(Xiang et al, 2001). In rainbow trout cell lines, apoptosis was shown to be the most prominent cell death mechanism after Cd exposure although others such as necroptosis were also strongly suggested (Krumschnabel et al., 2010). In zebrafish, the ZFL cell line exposed to Cu showed down-regulated *bcl2* and up-regulated *bax* gene expression, demonstrating apoptosis cell death (Sandrini et al., 2009), as our data. At molecular level, members of the pro- or anti-apoptotic regulators have been shown to be altered in fish exposed to metals and mediating apoptosis after oxidative stress (Choi et al., 2010; Risso-De Faverney et al., 2004; Zheng et al., 2014). Thus, our data suggest that oxidative stress might be responsible for the alteration of apoptosis-related regulators *bcl2* and *bax* genes and, finally, SAF-1 cell apoptosis.

Chapter 3

Cytotoxicity and alterations at transcriptional level caused by metals on fish erythrocytes *in vitro*

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Abstract

The *in vitro* use of fish erythrocytes to test the toxicity of aquatic pollutants could be a valuable alternative to fish bioassays but has received little attention. In this study, erythrocytes from marine gilthead seabream (*S. aurata)* and European sea bass (*D. labrax*) specimens were exposed for 24 h to Cd, Hg, Pb and As and the resulting cytotoxicity was evaluated. Exposure to metals produced a dose-dependent reduction in the viability, and mercury showed the highest toxicity followed by MeHg, Cd, As and Pb. Moreover, fish erythrocytes incubated with each one of the metals exhibited alteration in gene expression profile of *mta*, *sod*, *cat*, *prx*, *gr*, *hsp70* and *hsp90*, *bcl2* and *calp1* indicating cellular protection, stress and apoptosis death as well as oxidative stress. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish erythrocytes *in vitro*.

1. Introduction

Anthropogenic actions have resulted in an increased flux of metallic substances into the aquatic environment (Yang and Rose, 2003), a fact that has led to the investigation of the effect of metals and metalloids on the biological functions of marine organism such as fish. Among the adverse effects, metals can produce mortality or alterations in blood, metabolism, nutrition, reproduction, development and immunity (Bols et al., 2001; Di Giulio and Hinton, 2008; Sweet and Zelikoff, 2001). Some studies have shown that aquatic pollutants including metals alter fish haematological indices (haematocrit, red blood cell count per unit blood volume and haemoglobin (Hb) concentration), as well as blood/plasma ions, hormones, metabolites, proteins or enzymes (Schlenk et al., 2008). However, there are not many papers dealing with the toxicological effects on fish blood cells, namely erythrocytes. *In vitro* toxicological tests are gaining traction as alternatives to *in vivo* tests because they are more cost- and time- effective and have fewer ethical issues. The fact that fish erythrocytes are nucleated, contain organelles that exhibit good resistance in primary cultures and are easily handled, constitutes a very interesting cellular model for toxicological studies *in vitro* (Bogé and Roche, 1996). Furthermore, experimental fish are not killed which turns them into a valuable alternative to fish bioassays and contributes to the three R's. In addition, erythrocytes are targets for metals and used to distribute them along the body. Owing to the high oxygen and iron concentrations in the cytoplasm, erythrocytes can continuously produce ROS as a result of haemoglobin oxidation to methaemoglobin (Çimen, 2008; Giulivi and Davies, 2001), and therefore are exposed to potential oxidative stress. To maintain the ROS balance, as in mammals, fish erythrocytes are well protected by radical scavengers, including enzymatic and nonenzymatic systems. The enzymatic systems in erythrocytes consist of mitochondrial and cytosolic SOD, CAT, GPx and PRXs (Çimen, 2008; Scott et al., 1989). *In vivo* toxicological studies in fish erythrocytes have reported the effects of various chemicals in the morphology, haemolysis, nuclear deformation, amitosis (Bogé and Roche, 1996; Witeska, 2013) and genotoxic damage (Bagdonas and Vosylienė, 2006; Monteiro et al., 2011). However, fewer studies have evaluated the toxicological role of metals on fish erythrocytes *in vitro*, which could provide basic information on the nature of the tested agents and/or the cellular response Thus, rainbow trout (*Oncorhynchus mykiss*) naïve erythrocytes exposed *in vitro* to Cu showed little effect on

ROS production (Fedeli et al., 2010) whilst exposure to titanium dioxide nanoparticles produced cytotoxicity and DNA damage but not ROS production (Sekar and Falcioni, 2014). In the case of a neotropical freshwater fish (*Prochilodus lineatus*), erythrocyte exposure to lead also confirmed genotoxic and cytotoxic effects (Monteiro et al., 2011). Nevertheless, very few papers have evaluated the specific cellular responses of marine fish erythrocytes against pollutants at protein or messenger RNA (mRNA) levels (Roche and Bogé, 1993). To our knowledge, only one study (Fulladosa et al., 2006) has evaluated the MT expression *in vitro* in fish erythrocytes, showing a similar trend to that recorded in different fish species and models (Carbonell et al., 1998; Morcillo et al., 2015a, b, 2016). MTs, together with HSPs, are important proteins involved in cellular protection and concretely in the protection against metals (Bourdineaud et al., 2006; Morimoto, 2011). ROS induction by metals provokes DNA damage and apoptosis, but no studies have been found in the literature about the cell death mechanism after metal exposure in fish erythrocytes. In humans, it has been described a particular erythrocyte cell death mechanism called eryptosis that resembles to apoptosis of nucleated cells (Lang et al., 2006). Some differences between eryptosis and apoptosis reside in the fact that mammalian erythrocytes lack nuclei and mitochondria and that the molecular signalling pathways are not identical (Lang et al., 2012). Eryptosis occurs under natural conditions in erythrocytes, and eryptotic cells are engulfed and degraded by macrophages contributing to the animal haemostasis although it also occurs under pathophysiological situations. Among these situations, eryptosis has been demonstrated on human erythrocytes exposed to Cd, Hg, Pb, or As (Eisele et al., 2006; Kempe et al., 2005; Mahmudet al., 2009; Sopjani et al., 2008). In addition, a report has demonstrated the role of B cell lymphoma-2 (Bcl2) and Bcl-2 homologous antagonist killer (Bak) proteins in the human erythrocyte survival *in vitro* (Walsh et al., 2002), but no studies were found in fish erythrocytes.

Taking into consideration that pollutants affect fish hematological parameters and the scarce studies evaluating their effects on fish erythrocytes at molecular level, we aimed to evaluate the cytotoxicity of metals (Cd, Hg, Pb) and a metalloid (As) as well as the transcription pattern of genes related to cellular oxidative stress, protection, and death after 24 h exposure in two teleost fish species: gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*), the most important Mediterranean cultured fish species.

2. Material and methods

2.1. Animals

Thirty specimens of 80-100 g body weight of the seawater teleost gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*) obtained from local fish farms, were kept in seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia (Spain) The water was maintained at 20 ± 2°C, with a flow rate of 900 L/h, and 28‰ salinity. The photoperiod was 12 h light/12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight/day. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Erythrocyte isolation

Fish were taken from the aquaria and 200 µl of blood was immediately withdrawn into a heparinized syringe from the caudal vein and placed into 4 ml of PBS (phosphate buffered saline containing 0.35% sodium chloride, to adjust the medium's osmolarity) with 10 mM glucose, and the fish were returned to the aquaria. Blood was layered over a 51% Percoll density gradient (Pharmacia) and centrifuged (400 ×g for 30 min, 4 ºC) to separate leucocytes and erythrocytes, which were located in the pellets, collected, washed twice with PBS, counted and adjusted to 5×10^8 cells/mL in PBS with 10 mM glucose.

2.3. Metals exposure

Different salts of the tested metals (Sigma-Aldrich) were used: Cd chloride ($CdCl_2$), MeHg (II) chloride [CH_3HgCl (MeHg)], mercury (II) chloride ($HgCl_2$), lead (II) nitrate ($Pb(NO_3)_2$) and trioxide As (As_2O_3). Each salt was initially dissolved in PBS with 10 mM glucose and dilutions for each concentration were daily prepared. Prior to carrying out the assays, the osmolarity of these solutions was measured in an osmometer (Wescor) to avoid effects due to this parameter.

For each individual fish, 100 µL of blood erythrocytes were placed into separate 1.5 ml Eppendorf tubes in triplicate and exposed with 1 ml of PBS with 10 mM glucose (controls) or metal solutions, to make final concentrations of 10-100 µM for Cd, 5-75

µM for MeHg, 1-10 µM for Hg, 0.1-3 mM for Pb and 10-500 µM for As. Samples were gently shaken in an orbital agitator (24 h at 24 ºC). Erythrocytes from 6 different and independent fish were used for cytotoxicity curves and from 4 for gene expression studies.

2.4 Cytotoxicity assays

2.4.1. Propidium iodide (PI) uptake

In order to determinate the viability of the seabream and sea bass erythrocytes; we assessed the abundance of dead erythrocytes using a flow cytometry technique (Ormerod, 1990). Following 24 h of metal exposure, 10 µL of each sample was transferred to 5 mL tubes (Becton Dickinson) containing 400 µL of PBS and 100 µL of PI (400 µg/mL; Sigma-Aldrich). All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10,000 cells, which were acquired at a rate of 300 cells/s. Data were collected in the form of two-parameter side scatter (SSC, granularity) and forward scatter (FSC, size), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were constructed on a computerised system. With this method dead (PI^+) and viable (PI^-) cells were discriminated and analysed.

2.4.2. Oxyhemoglobin release

Erythrocyte exposure to metals results in cell death and liberation of their content being haemoglobin, in either oxyhemoglobin (HbO_2) or deoxyhemoglobin forms, the most abundant protein. Thus, the viability of the cells was determined by the hemolysis of erythrocytes and consequent liberation of the HbO_2 to the medium (De Kretser and Waldron, 1963; Jan and Frantisek, 2000; Martínez-López et al., 2005). After 24 h of exposure, the samples were centrifuged (10,000 × g, 1 min) and 100 µl of the supernatant transferred to 96 flat-bottomed well plates (Nunc) and the absorbance at 542 nm (the maximum absorbance for HbO_2) analysed in a plate reader (BMG, Fluoro Star Galaxy). Positive (maximum hemolysis and absorbance) or negative (minimum hemolysis and absorbance) controls consisted on 100 µL of erythrocytes in 1 mL of sterile distilled water or in 1 mL PBS with 10 mM glucose, respectively.

For each method, cell viability data and the metal concentrations were represented and fitted with an exponential decay 3 parameter curve [f = y 0 + a × exp (-bx)]. Fitted curves always showed r^2 values higher than 0.96 which are therefore the only ones presented in the cytotoxicity curves. The concentration producing 50% cell death (EC_{50}) was determined for all metals and assays using SigmaPlot software. According to the PI method, EC_0 (the minimum concentrations used and that failed to be cytotoxic) and EC_{50} were used in this study as the concentrations to measure gene expression.

2.5. Gene expression analysis by real-time PCR

After 24 h of erythrocytes exposure to the EC_0 or EC_{50} of the metals samples were centrifuged, the supernatants were aspirated and TRIzol Reagent (Life Technologies) was added to the wells in order to extract the total RNA as indicated by the manufacturer. 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 any genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer.

The expression of genes involved in cellular oxidative stress (*sod*, *cat*, *gr* and *prx1*), cellular protection (*mta*, *hsp70* and 90) cellular apoptosis (*bax*) and eryptosis (*calp1*) was evaluated by real-time PCR with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 µL of 2 × SYBR Green supermix, 5 µL of primers (0.6 mM each) and 5 µL of cDNA template) were incubated (10 min, 95 ºC), followed by 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC, and finally 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample and calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). Negative controls had no amplification product and control templates showed no primer-dimer formations. Gene names follow the accepted nomenclature for zebrafish (https://wiki.zfin.org/). The primers used in the present study are shown in Table 5. In all cases, each PCR was performed with triplicate samples from four specimens.

Table 5. Primers used for analysis of gene expression by real-time PCR.

Gene name	Gene abbreviation	Fish specie	Acc. numbers	Sequence (5'-3')
Elongation factor 1-alpha	*ef1a*	Seabream	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG
		Sea bass	AJ866727	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG
Cu/Zn superoxide dismutase	*sod*	Seabream	AJ937872	CCATGGTAAGAATCATGGCGG
				CGTGGATCACCATGGTTCTG
		Sea bass	FJ860004	TGTTGGAGACCTGGGAGATG
				ATTGGGCCTGTGAGAGTGAG
Catalase	*cat*	Seabream	FG264808	TTCCCGTCCTTCATTCACTC
				CTCCAGAAGTCCCACACCAT
		Sea bass	FJ860003	GAGGTTTGCCTGATGGCTAC
				TGCAGTAGAAACGCTCACA
Glutathione reductase	*gr*	Seabream	AJ937873	CAAAGCGCAGTGTGATTGTGG
				CCACTCCGGAGTTTTGCATTTC
		Sea bass	FM020412	TGCACCAAAGAACTGCAGAA
				ACGAGTGTCACCTCCAGTCC
Peroxiredoxin 1	*prx1*	Seabream	GQ252679	CTCCAAGCAATAATAAGCCCAAAG
				TCACTCTACAGACAACAGAACAC
		Sea bass	AM987213	CTGCCGAAGATTTCAGGAAGA
				CGCCGTGTGTCAGATACCAG
Metallothionein-A	*mta*	Seabream	X97276	ACAAACTGCTCCTGCACCTC
				CAGCTAGTGTCGCACGTCTT
		Sea bass	AF199014	GCACCACCTGCAAGAAGACT
				AGCTGGTGTCGCACGTCT
Heat shock protein 70	*hsp70*	Seabream	EU805481	AATGTTCTGCGCATCATCAA
				GCCTCCACCAAGATCAAAGA
		Sea bass	AY423555	CTGCTAAGAATGGCCTGGAG
				CTCGTTGCACTTGTCCAGAA
Heat shock protein 90	*hsp90*	Seabream	DQ524994	GGAGCTGAACAAGACCAAGC
				AGGTGATCCTCCCAGTCGTT
		Sea bass	AY395632	CTCAGGGACAACTCCACCAT
				CTTTGTCGTTCTTGTCAGCA
Bcl-2 associated X protein	*bax*	Seabream	AM963390	CAACAAGATGGCATCACACC
				TGAACCCGCTCGTATATGAAA
		Sea bass	FM011848	TGTCGACTCGTCATCAAAGC
				CACATGTTCCCGGAGGTAGT
Calpain1	*calp1*	Seabream	KF444899	GTGGAGCTCCTCGCTGTATC
				GCTTGAACTGAGGGTTGAGC
		Sea bass	FJ821591	TGCAGAGGGGACTGAGTTCT
				CCGCCTGTAAAGTCCTCAAA

2.6. Statistical analysis

Data of the cytotoxic effects are presented for each metal and method using the fitted curves. Gene expression is expressed as fold change with respect to the control samples where values higher than 1 indicate up-regulation and lower than 1 down-regulation of each gene. EC_{50} values for each fish species and method were analysed by one-way analysis of variance (ANOVA; $P \leq 0.05$) to determine differences among metals, followed by a SNK (Student-Newman-Keuls) comparison mean test. Gene expression data were statistically analysed by two-way ANOVA ($P \leq 0.05$) to determine differences between control and metals and between the two fish species. Normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. A nonparametric Kruskal–Wallis test was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPPS 20.0 software (SPSS).

3. Results

3.1. Cytotoxicity assays

After 24 h exposure to metals, the viability of gilthead seabream and European sea bass erythrocytes declined in a dose-dependent manner compared to controls (Figure 20). The data were used to fit curves in order to identify the EC_{50} (Figure 20, Table 6). According to the PI method, EC_{50} values for seabream erythrocytes were of 22, 21, 2.4, 523 and 134 μM, and for sea bass 80, 28, 3.6, 1,100 and 190 μM for Cd, MeHg, Hg, Pb and As, respectively (Table 6). Hg was the most toxic metal for erythrocytes in both species followed by MeHg, Cd, As and Pb (Table 6). Cytotoxicity curves followed different shapes between fish species when the erythrocytes were exposed to Cd or MeHg. Thus, seabream erythrocytes are more resistant to Cd, Pb and As whilst the susceptibility to MeHg and Hg was roughly the same. PI uptake and HbO_2 release techniques revealed almost identical cytotoxicity curves except for Hg-exposed sea bass erythrocytes (Figure 20), which is also evidenced for the EC_{50} values (Table 6).

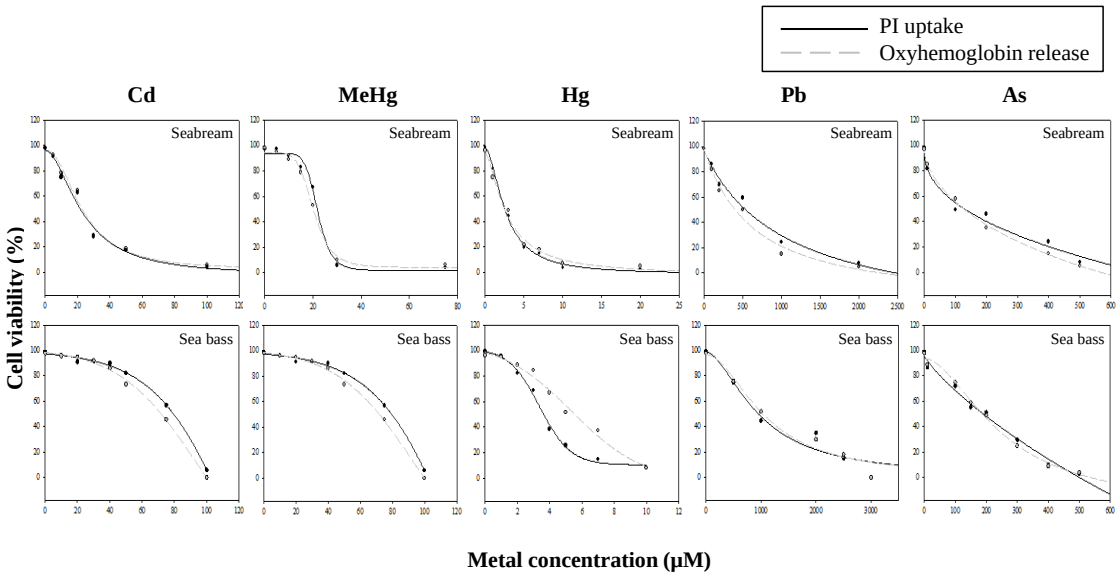

Figure 20. Cytotoxicity curves of gilthead seabream or European sea bass erythrocytes exposed to different metals for 24 h. Lines represent the fitted curve for each method: PI and HbO_2 release.

Table 6. Comparing the cytotoxicity of metals to fish erythrocytes from two species by two methods. Values of EC_{50} (µM; mean ± SEM; n = 6 independent fish) of fish erythrocytes after exposure to metals for 24 h. r^2 values of the fitted curves was higher than 0.96 in all cases. Statistically significant differences (ANOVA; P≤0.05) within each column (differences among metals) or row (differences between fish species and method for each metal) were denoted by different lower case or capital letters, respectively.

Metal	Gilthead seabream erythrocytes		European sea bass erythrocytes	
	PI uptake	Oxyhemoglobin release	PI uptake	Oxyhemoglobin release
Cd	22 ± 3.62^{aA}	23 ± 4.5^{aA}	80 ± 12.7^{aB}	71 ± 9.27^{aB}
MeHg	21 ± 1.06^{aA}	20 ± 0.71^{aA}	28 ± 0.76^{bAB}	29 ± 0.5^{bB}
Hg	2.4 ± 0.22^{bA}	2.7 ± 0.45^{bB}	3.6 ± 0.01^{cC}	$5.5 \pm 0.\ 3^{cD}$
Pb	523 ± 103^{cA}	374 ± 64^{cB}	$1{,}100 \pm 129^{dC}$	$1{,}000 \pm 201^{dC}$
As	134 ± 0.28^{dA}	129 ± 0.14^{dB}	190 ± 51^{aC}	189 ± 3^{eC}

3.2. Metals generate oxidative stress in erythrocytes

Exposure of erythrocytes to the EC_{50} of the metals for 24 h provoked oxidative stress (Figure 21, Supplementary data Figure 2.S1). First, all the metals, except Cd in sea bass,

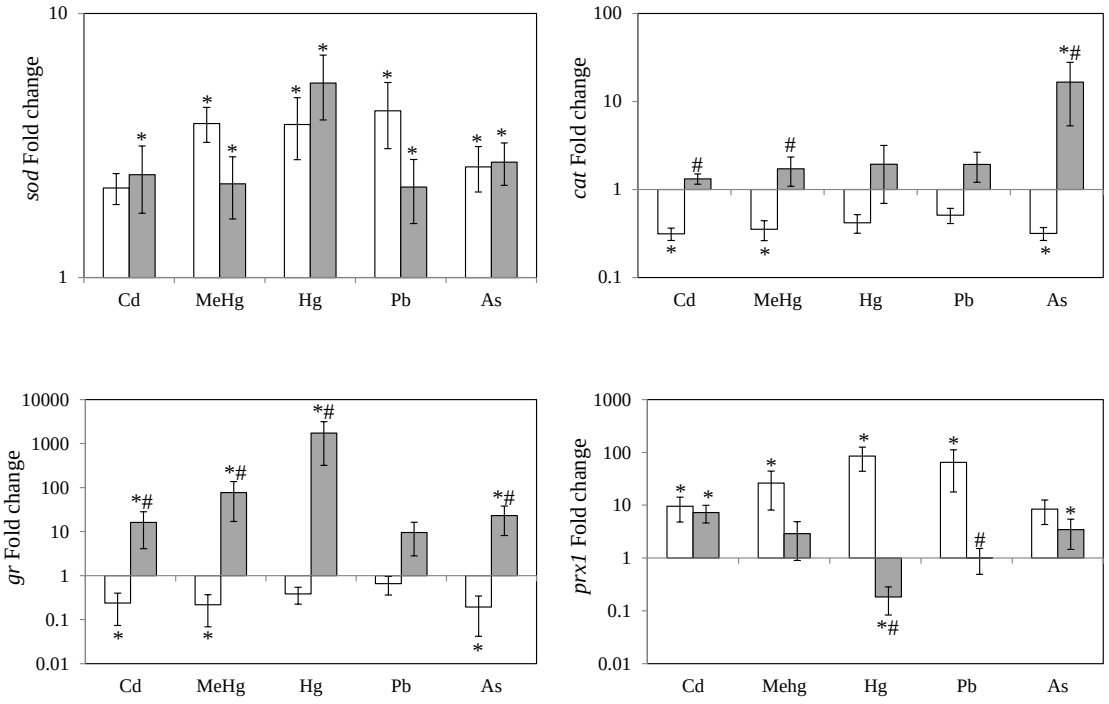

Figure 21. Expression of genes related to oxidative stress (*sod, cat, gr and prx1*) in gilthead seabream (white bars) or European sea bass (grey bars) erythrocytes exposed to the EC_{50} of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. Bars represent the mean ± SEM from 4 independent fish. Statistically significant differences ($P \leq 0.05$) between control and metal-exposed (*) and between the two species (#) were denoted.

significantly up-regulated the *sod* transcription in erythrocytes but no differences were observed between species. Surprisingly, *cat* and *gr* gene expression was always down-regulated after EC_{50} metal exposure of seabream erythrocytes, but statistically significant for Cd, MeHg and As. By contrast, they were always up-regulated in sea bass erythrocytes being only significant the *cat* gene for EC_{50} of As and *gr* for EC_{50} of MeHg, Hg and As. Significant effects between the two fish species were also observed. The *prx1* gene expression was significantly enhanced by EC_{50} of Cd, MeHg, Hg and Pb in seabream erythrocytes while only Cd and As exposure induced its transcription and

Hg down-regulated, in sea bass erythrocytes (Figure 21). As consequence, Hg and Pb effects were significantly different in the two fish species. Interestingly, exposure of erythrocytes to EC_0 provoked roughly the same profile at transcriptional level than the EC_{50} (Figure 2.SI).

3.3. Cellular protection is differently altered in seabream and sea bass erythrocytes

We evaluated the cellular protection to metals by the expression of the *mta* and *hsp70* and *hsp90* (Figure 22, 2.SI). As expected, EC_{50} of Cd or MeHg exposure of erythrocytes

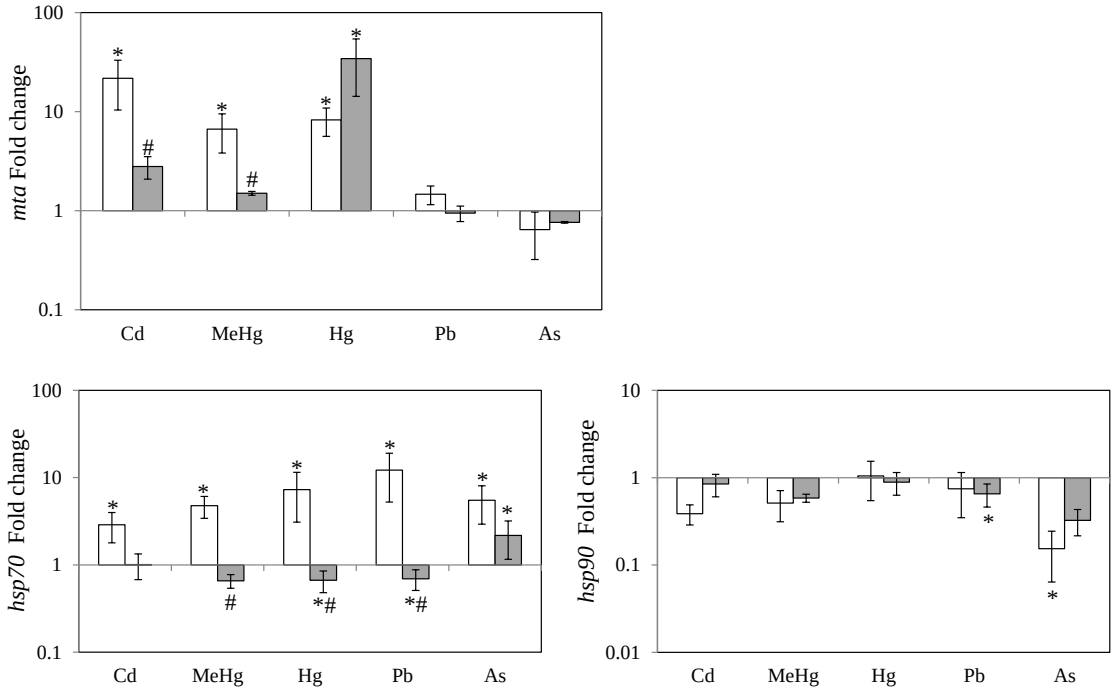

Figure 22. Expression of gene related to cell protection (*mta*, *hsp70* and *hsp90*) in gilthead seabream erythrocytes (white bars) or European sea bass (grey bars) erythrocytes after exposure to the EC_{50} of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. Bars represent the mean ± SEM from 4 independent fish. Statistically significant differences ($P<0.05$) between control and metal-exposed (*) and between the two species (#) were denoted.

from both fish species significantly up-regulated *mta* transcription (Figure 22). Surprisingly, exposure to EC_{50} of Pb or As did not alter *mta* mRNA abundance in the seabream and sea bass erythrocytes. All the metals increased *hsp70* gene expression in

seabream erythrocytes; however, Hg and Pb significantly down-regulated it in sea bass erythrocytes although As up-regulated it. Thus, exposure to MeHg, Hg and Pb provoked significant differences between gene expressions in erythrocytes for the two fish tested species. In contrast, most of the metals down-regulated the transcription of *hsp90* but only Pb and As did to a significant extent in seabream and sea bass erythrocytes, respectively (Figure 22).

As above, exposure of seabream or sea bass erythrocytes to EC_0 of metals induced a very similar transcriptomic profile as the EC_{50} (Figure 2.SI).

3.4. Metals induce apoptosis and eryptosis cell death

The expression of typical markers for apoptosis (pro-apoptotic Bcl2-associated X gene, *bax*) and eryptosis (μ-calpain, *calp1*) was assayed in fish erythrocytes (Figure 23, 2.SI).

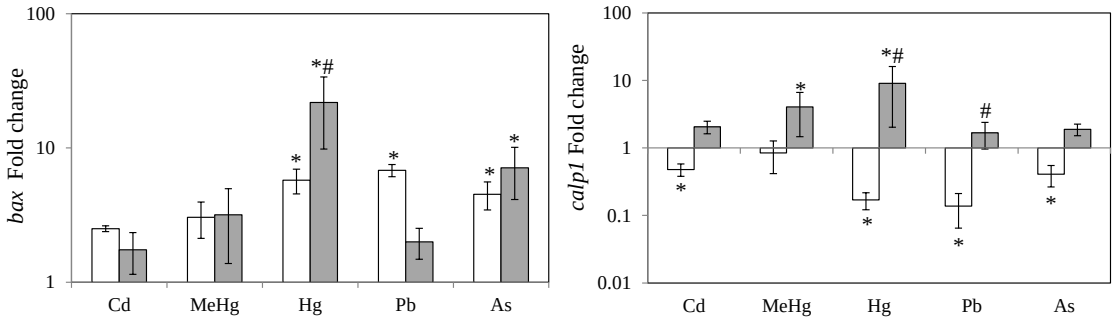

Figure 23. Expression of genes related to cell death (*bax* and *calp1*) in gilthead seabream (white bars) or European sea bass (grey bars) erythrocytes exposed to the EC_{50} of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. Bars represent the mean ± SEM from 4 independent fish. Statistically significant differences ($P<0.05$) between control and metal-exposed (*) and between the two species (#) were denoted.

Seabream and sea bass erythrocytes exposed to EC_{50} of Hg, Pb and As induced an up-regulation of *bax* transcripts, demonstrating the apoptosis cell death process. On the other hand, EC_{50} of Cd and MeHg did not alter it to a significant level. Surprisingly, *calp1* gene expression was greatly impaired by all EC_{50} of metals, except MeHg in seabream erythrocytes; however, exposure to MeHg or Hg produced a general induction

of *calp1* mRNA transcription in sea bass erythrocytes (Figure 22), which could be related to the eryptosis process (Figure 22) (Lang et al., 2006).

Finally, the effects provoked in fish erythrocytes at gene expression level by EC_0 and EC_{50} metals were comparable (Figure 2.SI).

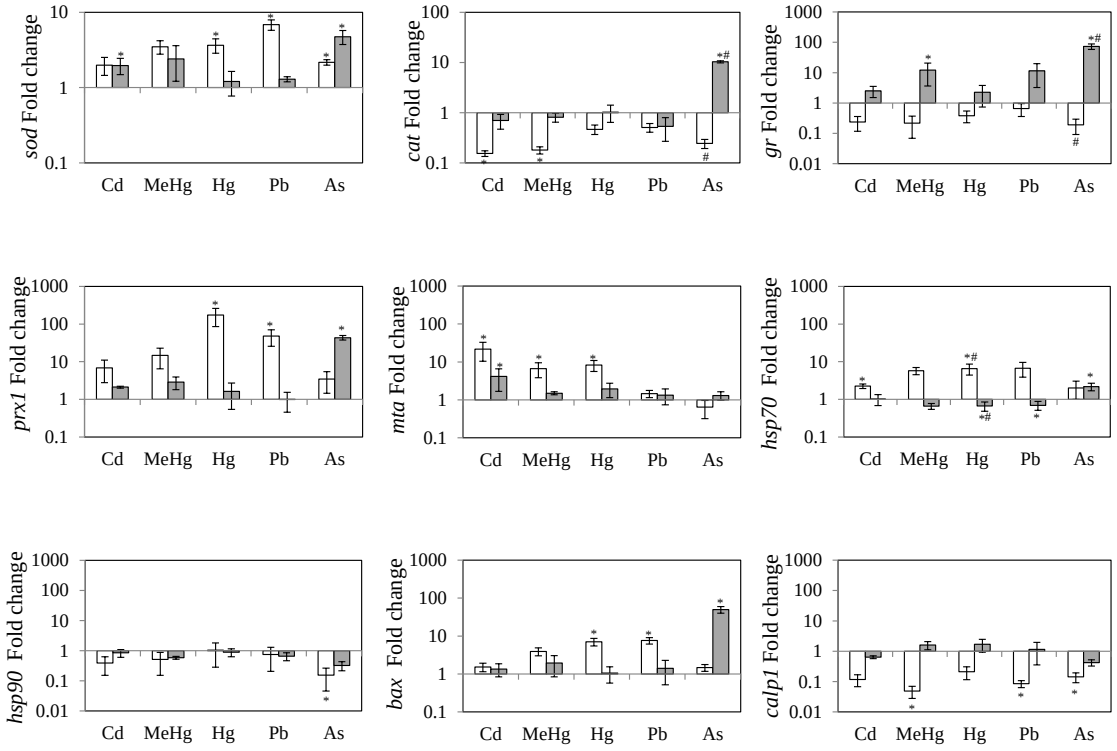

Figure 2.SI. Expression of genes related to oxidative stress (*sod, cat, gr and prx1*), cell protection (*mta, hsp70, hsp90*) and cell death (*bax and calp1*) in gilthead seabream (white bars) or European sea bass (grey bars) erythrocytes exposed to the EC_0 of each metal for 24 h. Data are expressed as fold change with respect to the control erythrocytes. Bars represent the mean ± SEM from 4 independent fish. Statistically significant differences ($P \leq 0.05$) between control and metal-exposed (*) and between the two species (#) were denoted.

4. Discussion

Fish erythrocytes could be useful for toxicological studies of aquatic pollutants because they remain viable in primary cultures and are easy to obtain and manipulate. However, very few papers have evaluated the toxicological effects on fish erythrocytes and those available include morphological changes (Bogé and Roche, 1996; Monteiro et al., 2011;

Witeska, 2004), genotoxic damage (Bagdonas and Vosylienė, 2006; Mitchelmore and Chipman, 1998) and MT expression (Fulladosa et al., 2006). Among the cytotoxicity tests used *in vitro*, flow cytometry and spectrophotometry methods were used to evaluate the cell death of fish erythrocytes. The cytotoxicity assays used revealed comparable profiles, showing a dose-dependent curve after 24 h exposure to the metals in erythrocytes from gilthead sea bream and European sea bass. The EC_{50} values showed that toxicity after 24 h exposure to metals was Hg>MeHg>Cd>As>Pb for erythrocytes from both species. However, sea bream erythrocytes were more sensitive to the metals than sea bass erythrocytes, showing lower EC_{50}. Moreover, we have shown for the first time that the oxyhaemoglobin release method could be used in fish ecotoxicological testing and is more sensible than the PI uptake. In addition, it has the advantage of using unsophisticated and affordable equipment and no need for reagents such as PI, which is carcinogenic. This method has been satisfactorily used in toxicological studies conducted in humans, rats and birds (De Kretser and Waldron, 1963; Jan and Frantisek, 2000; Martínez- López et al., 2005) and should be further explored in fish. Cytotoxic effects of metals observed on fish erythrocytes were compared to the few studies available in the literature. For instance, sea bream erythrocytes exposed to 100 µM Hg for 1 h showed a 6.1 % of haemolysis (Gwozdzinski et al., 1992) while this dose for 24 h showed 100 % haemolysis, which could be attributed to a rapid and adverse effect of Hg upon membrane integrity. Human erythrocytes exposed to 10 mM As (III) for 5 h exhibited 0.7 % haemolysis (Shannon and Winski, 1998). Thus, As seems to show a slower and lower toxicity mechanism than Hg, which is also observed in our results. Studies using bird erythrocytes reported a lower EC_{50} for Cd (0.027 mM) than for lead (1.84 mM) (Hernández-García et al., 2014), which also agrees with our data. Other studies in the RTG-2 fish cell line confirmed the highest toxicity of Hg compared to other metals (Maracine and Segner, 1998), whilst in the case of the grass carp (*Ctenopharyngodon idella*) ZC-7901 cell line, the order was Cd^{2+}>Hg^{2+}>Pb^{2+}>Cu^{2+}>Cr^{6+}>As^{5+} (Xiang et al., 2001). Moreover, in the rainbow trout RTL-W1 (derived from the liver) EC_{50} values for Cd are quite similar to our data (Dayeh et al., 2005). Differences in the fish environment (fresh or marine water), as well as the metal form, solubility, exposure time, interactions with culture medium components, etc., could be responsible for some of the differences found with those mentioned in the literature. In fact, it is known that culture medium composition

or percentage of serum supplementation affects the metal cytotoxicity (Borenfreund and Puerner, 1985; Dayeh et al., 2005; Segner, 1998). For example, differences between culture medium could be behind the low EC_{50} values observed in this study (PBS with 10 mM glucose) and the higher values observed in the SAF-1 cell line (derived from fins of gilthead sea bream and exposed in L-15 with 10 % serum) (Morcillo et al., 2016), that would be further investigated. Very few studies concerned with the erythrocyte toxicology of metals at the gene level are found in the literature. Thus, we have evaluated the transcription of some important genes involved in oxidative stress, cell protection and death after 24 h of exposure to the EC_0 or EC_{50} of each metal. With respect to oxidative stress, we found that all the metals induced a significant up-regulation of *sod* gene expression in sea bream and sea bass erythrocytes. In sea bass, erythrocytes exposed to 10– 100 μM Hg for 1 h the SOD enzyme activity increased in all doses (Gwozdzinski et al., 1992), which agrees with our results. However, the decrease in SOD activity of sea bass erythrocytes by Cu^{2+} and Zn^{2+} was unexpected (Roche and Bogé, 1993), since these metallic ions are potent ROS activators. *In vivo* experiments with goldfish (*Carassius gibelio*) erythrocytes evaluating SOD activity showed a significant decrease after the first day of Cd exposure or an increase after 7 or 15 days (Zikić et al., 2001). This increased gene and protein SOD could be involved, as demonstrated in human erythrocytes, in the prevention of methaemoglobin formation (Dumaswala et al., 1999). In sea bream erythrocytes, both *cat* and *gr* transcriptions were significantly down-regulated, but in the case of sea bass, *cat* was up-regulated by As and *gr* by most metals. In sea bass, erythrocyte exposure to Cr or Zn increased CAT activity while GR activity was decreased by Cr, Cu and Zn exposure (Roche and Bogé, 1993). These opposite effects between sea bream and sea bass erythrocytes were not observed in freshly isolated head-kidney leucocytes (Morcillo et al., 2015a, b). Contradictory results were also found in the literature. For example, CAT activity was increased or decreased after Cd exposure of fish erythrocytes (Firat and Kargin, 2010) and GR activity was reduced after Pb exposure in human erythrocytes *in vitro* or *in vivo* (Hunaiti et al., 1995; Hunaiti and Soud, 2000). In rat erythrocytes exposed to As, the CAT activity remained unchanged (Dwivedi and Flora, 2015). It was previously reported that reduced nicotinamide adenine dinucleotide-hydrogen (NADH) plays an important role in the activation of CAT from its inactivated form (Das et al., 2010) and insufficient supply of NADH during arsenic metabolism might decrease the activity of

CAT (Kirkman et al., 1987). Special attention should be focused on the increase (>1000-fold) of the *gr* transcription after Hg exposure in sea bass erythrocytes. Similarly, European sea bass erythrocytes exposed to low $HgCl_2$ concentrations for 1 h resulted in increased SOD, CAT, peroxidase and glutathione peroxidase activities, but not in the case of human red blood cells, suggesting the presence of ROS and its partial elimination (Gwozdzinski et al., 1992). Similarly, *in vivo* exposure to sublethal dosages of Hg resulted in increased metabolism of glutathione and its associated enzymes in an effort to control the ROS production (Elia et al., 2003). In contrast, inorganic mercury nitrate added *in vitro* to stroma free rat erythrocyte haemolysates resulted in a clear inhibition of GR activity (Mykkanen and Ganther, 1974). Thus, our data suggest that the overexpression of *gr* could imply that glutathione is in reduced (GSH) state and is ready to be oxidized by glutathione peroxidase and scavenge more ROS. The scavenging of H_2O_2 and peroxinitrites by Prx1 and Prx2 has been also demonstrated in erythrocytes (Dubuisson et al., 2004; Lee et al., 2003; Low et al., 2008; Neumann et al., 2003). In our study, *prx1* gene expression was intensely up-regulated by metals in sea bream erythrocytes; however, only Cd and As exposure induced *prx1* transcription in sea bass erythrocytes while Hg exposure down-regulated it. In another study, Cd, Hg forms and Pb exposure elicited the increase of *prx1* gene expression in the sea bream SAF-1 cell line but As decreased it (Morcillo et al., 2016). The antioxidant mechanisms (*cat* and *gr* gene expression) in sea bream erythrocytes were more inhibited than in sea bass erythrocytes and this could explain higher sensitivity of sea bream erythrocytes to metal exposure. As in mammals, MTs and HSP are relevant in the cellular protection of fish erythrocytes (Currie and Tufts, 1997; Ferencz and Hermesz, 2015). Present results show an increase of the *mta* gene expression after Cd and Hg forms while Pb or As exposure did not alter it in sea bream or sea bass erythrocytes. The finding that Cd and Hg are transported in the blood bound to MTA cysteine residues (Goyer and Clarkson, 1996; Zalups, 2000) could partly explain our observations. In contrast, after 1 or 2 h exposure to sublethal concentrations of Cd and Pb in silver sea bream (*Sparus sarba*) *in vitro*, no overexpression of MTA was evidenced likely due to the short time exposure (Fulladosa et al., 2006). In common carp specimens exposed to Cd, the *mt* transcription was related with low adverse effects in the blood compared to those observed in skin (Ferencz and Hermesz, 2015). In the case of *hsp* gene expression, mRNA coding for stress proteins is actively produced in red blood cells of the brook trout (*Salvelinus*

fontinalis) summited to a heat shock (Lund et al., 2003) while we found differences in the gene expression and fish species. This result suggests a possible influence of the metal concentration and exposure time in the stress protein expression. Fulladosa et al. (2006) found that the maximal overexpression of HSP70 occurred after 3 h exposure to 20 μM Cd but also that prolonged exposure reduced it. In addition, they showed that increasing concentrations of Cd failed to further increase the HSP70 overexpression, while in the case of Cr and Pb, this was reduced. Therefore, the relation between ROS production and the oxidative stress mechanisms and cell protection deserves further investigation in fish erythrocytes. Finally, it is widely accepted that overproduction of ROS induced by metals provokes apoptotic cell death (Rana, 2008). In the case of human erythrocytes, these ROS provoke a suicidal erythrocyte death named eryptosis (a type of apoptosis that takes place in erythrocytes) (Föller et al., 2008; Kempe et al., 2005; Lang and Lang, 2015; Shin et al., 2007) that may involve stimulation of two proteases that play an essential role in apoptosis (caspases and calpain), with subsequent degradation of the cytoskeleton, but how erythrocyte cell death is regulated is still under debate. Walsh et al. (2002) have demonstrated the role of BAK and BCL2 proteins in the human erythrocyte survival *in vitro*, but no studies are found in fish erythrocytes in this respect. Fish erythrocytes possess nuclei and mitochondria, both absent in mature mammalian erythrocytes, and the last one are major players in the apoptosis cell death (Moyes et al., 2002). Thus, pro-apoptotic *bax* and μ- calpain (*calp1*) gene expression was assessed in sea bream and sea bass erythrocytes. As expected, a significant up-regulation of *bax* transcription after exposure to Hg, Pb and As in erythrocytes from both species suggested that these metals induced apoptosis cell death, which resulted positive to PI uptake and haemoglobin release after 24 h. However, no differences were observed after Cd or MeHg exposure in *bax* mRNA levels, so these metals could not trigger apoptosis, or alter other genes involved in the regulation of apoptosis in erythrocytes from both species. For example, Cd and MeHg can provoke necrosis or necroptosis cell death in fish cell lines or leucocytes (Kim and Sharma, 2004; Krumschnabel et al., 2005; Morcillo et al., 2015a; b; Rana, 2008; Selvaraj et al., 2013). In fact, excessive oxidative stress can induce necrosis (Çimen, 2008; Hong et al., 2009) and the conversion of apoptosis to necrosis in cultured cells (Higuchi and Yoshimoto, 2002). Transcription of *calp1* was strongly down-regulated after metal exposure in sea bream erythrocytes in contrast to sea bass erythrocytes. A possible reason could be the

fact that as a result of an oxidative stress situation, reduced glutathione (GSH) is depleted, which triggers activation of Ca^{2+}-permeable cation channels, provoking Ca^{2+} influx and activation of *μ-calpain* (Lang et al., 2006; Calderon-Salinas et al., 2010) Some of the main molecules involved in the eryptosis process demonstrated in the present study are summarized in Figure 5. In the case of sea bream erythrocytes, *gr* is down-regulated after metal exposure; thus, no GHS depletion and Ca^{2+} entry occurs, triggering an inactivation of *μ-calpain*, which is in accordance with the down-regulation of *calp1* in our study. Alternatively, an up-regulation of *gr* in sea bass erythrocytes could deplete GHS leading to the activation of *calp1*, at least at the gene level, which is in agreement with our results.

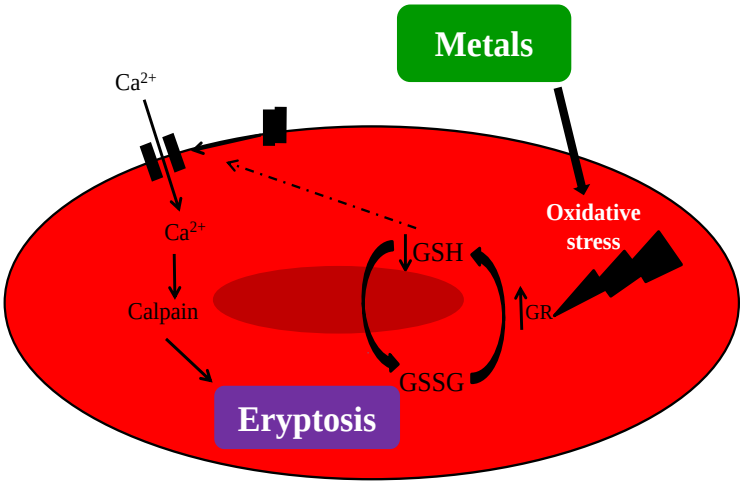

Figure 24. Synopsis of the mechanism and the signaling pathway involved in eryptosis. Modified from Lang et al. (2006). GR, glutathione reductase; GSH, glutathione: GSSG, glutathione disulfide.

Chapter 4

In vitro effects of metals on isolated head-kidney and blood leucocytes of the teleost fish *Sparus aurata* L. and *Dicentrarchus labrax* L.

Abstract

The *in vitro* use of fish leucocytes to test the toxicity of aquatic pollutants, and particularly the immunotoxicological effects, could be a valuable alternative to fish bioassays but has received little attention. In this study, head-kidney and peripheral blood leucocytes (HKLs and PBLs, respectively) from gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*) specimens were exposed for 24 h to Cd, MeHg, Pb or As being evaluated the resulting cytotoxicity. Exposure to metals produced a dose-dependent reduction in the viability, and MeHg showed the highest toxicity followed by Cd, As and Pb. Interestingly, leucocytes from European sea bass are more resistant to metal exposure than those from gilthead seabream. Similarly, HKLs are always more sensitive than those isolated from blood from the same fish species. Moreover, fish leucocytes incubated with metals exhibited alterations in gene expression profiles that were more pronounced in the HKLs in general, being Pb the metal provoking less effects. Concretely, genes related to cellular protection (*mta*), stress (*hsp70*) and oxidative stress *(sod, cat, gr*,) were, in general, down-regulated in seabream HKLs but up-regulated in seabream PBLs and sea bass HKLs and PBLs. In addition, this profile lead to the increase of expression in genes related to apoptosis (*bcl2* and *casp3*). Finally, transcription of genes involved in immunity (*il1b* and *ighm*) was down-regulated, mainly in seabream leucocytes. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish leucocytes *in vitro* and insight into the mechanisms at gene level.

1. Introduction

Industrial development has caused an increase of metals such as Hg, Pb, Cd or As in marine ecosystems, being detectable in water and organism including fish (Bayen et al., 2005; Colombo et al., 2004). These pollutants in the marine environment produce severe problems, especially because they persist in the environment, and fish show the ability to absorb and accumulate higher levels of metals in their tissue than the level of toxic concentration in their environment (Alyakoob et al., 1994), with a consequent negative influence on fish homeostatic mechanisms but also for human consumers. Concretely, some studies have showed the adverse effects of metals in fish including mortality, alterations in hematological parameters, metabolism, nutrition, reproduction, development and immunodeficiency (Bols et al., 2001; Di Giulio and Hinton, 2008; Sweet and Zelikoff, 2001). In light of increasing social and political pressure to use nonmammalian systems for predicting human health risks and the recent impetus to develop biomarkers for assessing the biological effects of environmental stress, more and deeper studies are needed to better understand chemical-induced effects on aquatic species. Apart from classical biomarkers of toxicity such as oxidative stress or lipid peroxidation, other studies have revealed the impact of metals on the ecosystem and organism health suggesting the importance of including more and new markers. In this regard, though the fish immune system is considered a nonspecific marker for environmental biomonitoring it has direct implications in individual health and population growth (Jolly et al., 2014).

In vivo studies in fish, usually by waterborne exposure to pollutants, have confirmed alterations in innate and acquired immune functions, such as respiratory burst, phagocytosis, lymphocyte proliferation or antibody levels as well as interfering with host resistance against infectious pathogens (Bols et al., 2001; Cuesta et al., 2011; Zelikoff et al., 1995). Despite the fact that fish may be negatively impacted by different kind of pollutants, little is known about the toxicological role of metals on the immune system and the oxidative stress in fish leucocytes *in vitro*, which could serve as good models for immunotoxicological studies. Previous studies carried out in our laboratory demonstrated alterations in the immune functions (phagocytosis, respiratory burst and transcription of immune-relevant genes) and overproduction of ROS of gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*) head-kidney leucocytes (HKLs)

after 30 min or 2 h of exposure to metals (Morcillo el al., 2015a, b), which is in agreement with previous studies in fish (Bennani et al., 1996; Ghanmi et al., 1989; Sarmento et al., 2004; Viola et al., 1996). Similarly, few papers have dealt with the toxicological effects of metals in peripheral blood leucocytes (PBLs). For instance, common carp PBLs exposed to Cr decreased the lymphocyte proliferation (Steinhagen et al., 2004) as it did Cd or Zn, but not Pb or Cu, with the respiratory burst activity (Witeska and Wakulska, 2007). In the case of red drum PBLs, exposure to subtoxic doses of Hg increased cell proliferation whilst toxic doses induced massive calcium flux and activation of tyrosine kinase activity (MacDougal et al., 1996). However, only few papers have dealt with the toxicological mechanisms caused by metals at gene level. Thus, the regulation of gene expression of genes related to oxidative stress, cell death, metal protection or immunity has been evaluated *in vitro* in sea bass PBLs (Vazzana et al., 2014) and HKLs (Morcillo et al., 2015a), seabream HKLs (Morcillo et al., 2015b) or rainbow trout (*Oncorhynchus mykiss*) HK macrophages (Teles et al., 2011). First, these have showed, in general, an up-regulation of apoptosis marker genes, such as Bcl-2 associated X protein (*bax*) or caspase 3 (*casp3*), and oxidative stress-related genes, such as *sod* and *cat* (Morcillo et al., 2015a, b) after HKLs exposure to metals. Regarding immunity, seabream HKLs showed variable up- or down-regulation of genes related to lymphocyte markers, pro-inflammatory cytokines, antiviral, antimicrobial peptides or respiratory burst that differed with the metal used (Morcillo et al., 2015a) whilst in the case of sea bass very few changes occurred after Hg exposure (Morcillo et al., 2015a). In trout macrophages, Cu exposure up-regulated the transcription of interleukin-1β (*il1b*), tumour necrosis factor-α (*tnfa*), interleukin-6 (*il6*), serum amyloid A (*saa*), NADPH oxidase, glutathione peroxidase and trout C-polysaccharide binding protein (*tcpbp*) (Teles et al., 2011). Therefore, more studies are needed to clarify the toxicological effects and mechanisms of metals in fish leucocytes since it is known that *in vitro* cytotoxicity assays with fish cell lines are very well correlated to acute lethality tests *in vivo* (Segner, 2004).

Thereby, the present study aimed to compare the cytotoxicity of metals (Cd, Hg, Pb) and a metalloid (As) on HKLs and PBLs isolated from two teleost fish species: gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*), the most important farmed fish species in the Mediterranean area. Thus, HKLs and PBLs were exposed to Cd, MeHg, Pb or As for 24 h and viability and transcription of genes related to cellular and

oxidative stress, protection, death and immunity was determined. Comparisons between source of leucocytes and fish species will be discussed towards their implication at research and toxicological level.

2. Material and methods

2.1. Animals

Twenty specimens of 80-100 g body weight of the seawater teleost gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*) obtained from local fish farms, were kept in seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia (Spain). The water was maintained at 20 ± 2°C, with a flow rate of 900 L/h, and 28‰ salinity. The photoperiod was 12 h light/12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight/day. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Leucocyte isolation

Fish were anaesthetised with 0.21 mM benzocaine (stock dissolved in 4% acetone) (Sigma) and samples were taken under sterile conditions. For HKL isolation, fish were bled from the caudal vein to avoid tissue contamination with erythrocytes and HK tissue was excised, cut into small fragments and transferred to 7 mL of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride, 100 IU/mL penicillin (Life Technologies), 100 mg/mL streptomycin (Life Technologies) and 5% foetal bovine serum (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 (1,500 rpm, 10 min), counted and adjusted to 10^7 cells/mL in sRPMI. For PBL isolation, 200 µl of blood was immediately withdrawn from the caudal vein with a heparinized syringe and fish were returned to aquaria. Blood was mixed with 4 mL of sRPMI and layered over a 51% Percoll density gradient (Pharmacia), centrifuged (400 g for 30 min at 4ºC) and PBLs, located in the interface, were collected, washed twice, counted and adjusted to 10^6 cells/mL in sRPMI. In all cases, leucocyte viability was determined by the trypan blue exclusion test and resulted higher than 98%.

2.3. Metals exposure

Different salts of the tested metals (Sigma) were used: Cd chloride ($CdCl_2$), MeHg (II) chloride [CH_3HgCl (MeHg)], lead (II) nitrate ($Pb(NO_3)_2$) and trioxide As (As_2O_3). Each salt was initially dissolved in sterile purified water (Milli-Q) and dilutions for each concentration were daily prepared. Prior to carrying out the assays, the osmolarity of these solutions was measured in an osmometer (Wescor) to avoid effects due to osmolarity.

For leucocyte treatments, 180 µL of freshly isolated HKLs and PBLs were dispensed in separate wells, always in triplicate, of flat-bottomed 96-well plates (Nunc). Then, 20 µL/wellof water (controls) or metal solutions, to make final concentrations of 50-5,000 µM for Cd, 5-100 µM for MeHg, 500-5,000 µM for Pb or 2,000-5,000 µM for As, were added. Cells were exposed for 24 h at 25ºC in an incubator. Leucocytes from 6 independent specimens (not pooled) and assayed separately (tested in different days) except in the gene expression studies that we used 4 independent fish specimens.

2.4 Cytotoxicity assays

2.4.1. PI (propidium iodide) uptake

In order to determinate the viability of the seabream and sea bass leucocytes, we assessed the abundance of dead HKLs and PBLs using a flow cytometry technique based on fluorochrome labelling (Ormerod, 1990). Following 24 h of metal exposure, samples were mixed by pipetting and 200 µL of each sample were transferred to 5 mL tubes (Becton Dickinson) containing 400 µL of phosphate buffered saline (PBS) and 100 µL of PI (400 µg/mL; Sigma-Aldrich). All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10,000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (SSC, granularity) and forward scatter (FSC, size), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerised system. With this method dead (PI^+) and viable (PI^-) cells were discriminated and analysed.

2.4.2. Data analysis

For each method, cell viability data and the metal concentrations were represented and fitted with an exponential decay 3 parameter curve [f = y0 + a×exp(-bx)]. Fitted curves always showed r^2 values higher than 0.96 which are therefore the only ones presented in the cytotoxicity curves. The concentration producing 50% cell death (EC_{50}) was determined for all metals and assays using Sigma plot software (Table 7). According to viability data obtained by the PI method, EC_0 (the minimum concentrations used and that failed to be cytotoxic) and EC_{50} values calculated using the fitted curves were used in this study to evaluate gene expression.

2.5. Expression of genes by real-time PCR

After 24 h of HKLs and PBLs exposure to metals samples were centrifuged, the supernatant was aspirated and TRIzol Reagent (Life Technologies) was added to the wells in order to extract the total RNA as indicated by the manufacturer. 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 any genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer.

In the present study, the expression of genes involved in cellular oxidative stress (*sod*, *cat*, *gr*), cellular protection (*mta* and *hsp70*), cellular apoptosis (*bax* and *casp3*) and immune-relevant genes (*ighm*, *il1b*) has been evaluated by real-time PCR (RT-PCR) with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures containing 10 µL of 2 × SYBR Green supermix, 5 µL of primers (0.6 mM each) and 5 µL of cDNA template were incubated for 10 min at 95 ºC, followed by 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC, and finally 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample and calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). Negative controls had no amplification product and control templates showed no primer-dimer formations. Gene names follow the accepted nomenclature for zebrafish (https://wiki.zfin.org/). The primers used in the present study are shown in Table 7. In all

cases, each PCR was performed with triplicate samples from four independent specimens.

Table 7. Primers used for analysis of gene expression by real-time PCR.

Gene name	Gene abbreviation	Fish specie	Acc. numbers	Sequence (5'-3')
Elongation factor 1-alpha	*ef1a*	Seabream	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG
		Sea bass	AJ866727	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG
Cu/Zn superoxide dismutase	*sod*	Seabream	AJ937872	CCATGGTAAGAATCATGGCGG
				CGTGGATCACCATGGTTCTG
		Sea bass	FJ860004	TGTTGGAGACCTGGGAGATG
				ATTGGGCCTGTGAGAGTGAG
Catalase	*cat*	Seabream	FG264808	TTCCCGTCCTTCATTCACTC
				CTCCAGAAGTCCCACACCAT
		Sea bass	FJ860003	GAGGTTTGCCTGATGGCTAC
				TGCAGTAGAAACGCTCACA
Glutathione reductase	*gr*	Seabream	AJ937873	CAAAGCGCAGTGTGATTGTGG
				CCACTCCGGAGTTTTGCATTTC
		Sea bass	FM020412	TGCACCAAAGAACTGCAGAA
				ACGAGTGTCACCTCCAGTCC
Metallothionein-A	*mta*	Seabream	X97276	ACAAACTGCTCCTGCACCTC
				CAGCTAGTGTCGCACGTCTT
		Sea bass	AF199014	GCACCACCTGCAAGAAGACT
				AGCTGGTGTCGCACGTCT
Heat shock protein 70	*hsp70*	Seabream	EU805481	AATGTTCTGCGCATCATCAA
				GCCTCCACCAAGATCAAAGA
		Sea bass	AY423555	CTGCTAAGAATGGCCTGGAG
				CTCGTTGCACTTGTCCAGAA
Bcl-2 associated X protein	*bax*	Seabream	AM963390	CAACAAGATGGCATCACACC
				TGAACCCGCTCGTATATGAAA
		Sea bass	FM011848	TGTCGACTCGTCATCAAAGC
				CACATGTTCCCGGAGGTAGT
Caspase3	*casp3*	Seabream	EU722334	CTGATCTGGATGGAGGCATT
				AGTAGTAGCCTGGGGCTGTG
		Sea bass	DQ345773	AATTCACCAGGCTTCAATGC
				CTACGGCAGAGACGACATCA
Immunoglobulin M heavy chain	*ighm*	Seabream	AM493677	CAGCCTCGAGAAGTGGAAAC
				GAGGTTGACCAGGTTGGTGT
		Sea bass	FN908858	AGGACAGGACTGCTGCTGTT
				CACCTGCTGTCTGCTGTTGT
Interleukin-1 beta	*il1b*	Seabream	AJ277166	GGGCTGAACAACAGCACTCTC
				TTAACACTCTCCACCCTCCA
		Sea bass	AJ269472	CAGGACTCCGGTTTGAACAT
				GTCCATTCAAAAGGGGACAA

2.6. Statistical analysis

Data of the cytotoxic effects are presented for each metal using the fitted curves. Gene expression is expressed as relative expression to the house-keeping gene. Data were statistically analysed by one-way analysis of variance (ANOVA; $P<0.05$) to determine differences among metal concentration, followed by a post-hoc Tukey test. Normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. A nonparametric Kruskal–Wallis test was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPPS 20.0 software (SPSS).

3. Results

Data regarding regulation of gene expression by metals are presented as relative expression for each gene, compared to the housekeeping gene, in a graph and grouped with the following order: metal, fish species and leucocyte source. Transcription in control, EC_0 and EC_{50} metal exposed fish leucocytes is presented. It is interesting to note that in most cases, expression in sea bass leucocytes is higher than in seabream leucocytes and that PBLs expression is also higher than HKLs.

3.1. Cytotoxicity assays

After 24 h exposure to metals, mortality of gilthead seabream and European sea bass leucocytes was induced in a dose-dependent manner compared to controls (Figure 25). The data were used to fit curves in order to identify the EC_0 and EC_{50} (Figure 25, Table 8). According to the PI method, EC_{50} values for seabream HKLs were of 110, 5, 2,800 and 218 μM, and for seabream PBLs 171, 12, 2,900 and 491 μM for Cd, MeHg, Pb and As, respectively (Table 8). On the other hand, EC_{50} values for HKL sea bass were of 150, 300, 3,400 and 2,700 μM, and for sea bass PBLs were of 2,600, 464, 4,200 and 3,800 μM for Cd, MeHg, Pb and As, respectively (Table 8). MeHg was the most toxic metal for leucocytes in both species followed by Cd, As and Pb (Table 8). Cytotoxicity curves followed different shapes between fish species and source when the leucocytes were exposed to metals. Overall, PBLs are always more resistant to metals than HKLs in both species and the EC_{50} values reveal that sea bass leucocytes are more resistant to metals than in seabream (Table 8).

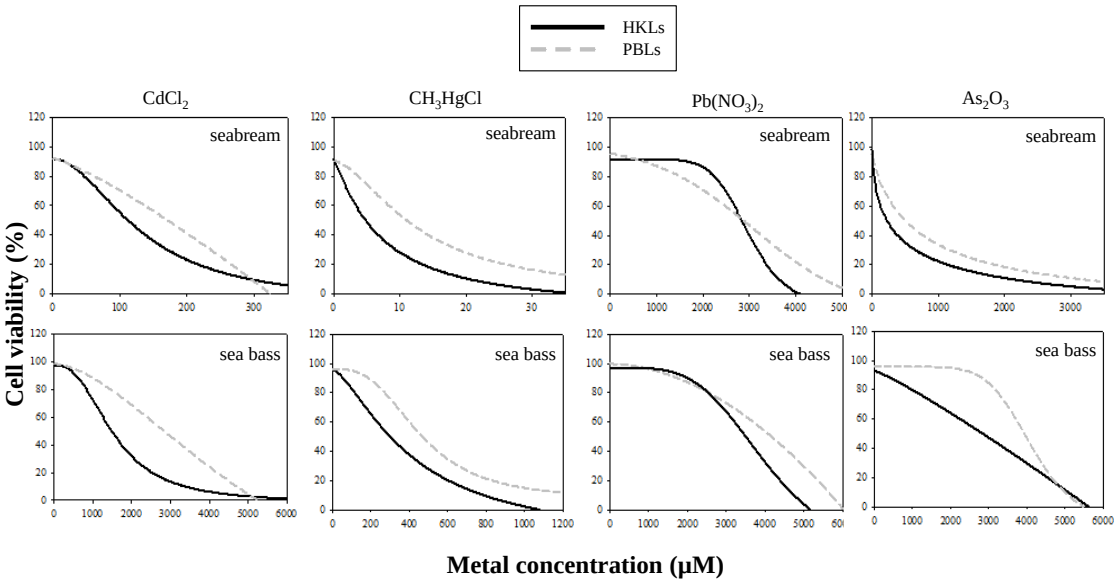

Figure 25. Cytotoxicity curves of gilthead seabream or European sea bass head-kidney (HKLs) of peripheral blood (PBLs) leucocytes exposed to metals for 24 h. Lines represent the fitted curve for each type of leucocyte sources.

Table 8. Values of EC_{50} (µM; mean ± SEM; n = 6 independent fish) of fish leucocytes after exposure to metals for 24 h. r^2 values of the fitted curves was higher than 0.96 in all cases. Different letters within each fish species and leucocyte source denote statistically significant differences (ANOVA; $P<0.05$).

Metals	HKLs		PBLs	
	seabream	**sea bass**	**seabream**	**sea bass**
$CdCl_2$	0.110 ± 0.028^{a}	1.5 ± 0.090^{a}	0.171 ± 0.034^{a}	2.6 ± 0.105^{a}
CH_3HgCl	0.005 ± 0.0006^{b}	0.3 ± 0.012^{b}	0.012 ± 0.0017^{b}	0.464 ± 0.038^{b}
$Pb(NO_3)_2$	2.8 ± 0.156^{c}	3.4 ± 0.124^{c}	2.9 ± 0.128^{c}	4.2 ± 0.301^{c}
As_2O_3	0.218 ± 0.014^{a}	2.7 ± 0.105^{a}	0.491 ± 0.065^{d}	3.8 ± 0.199^{d}

3.2. Antioxidant genes are down-regulated in seabream and up-regulated in sea bass leucocytes

Exposure of leucocytes to the EC_0 and EC_{50} of metals for 24 h provoked a regulation of the oxidative stress gene expression (Figure 26). Firstly, *sod* transcription was always significantly impaired by EC_0 or EC_{50} of Cd, MeHg, and As in seabream HKLs or after MeHg exposure in sea bass HKLs (Figure 26).

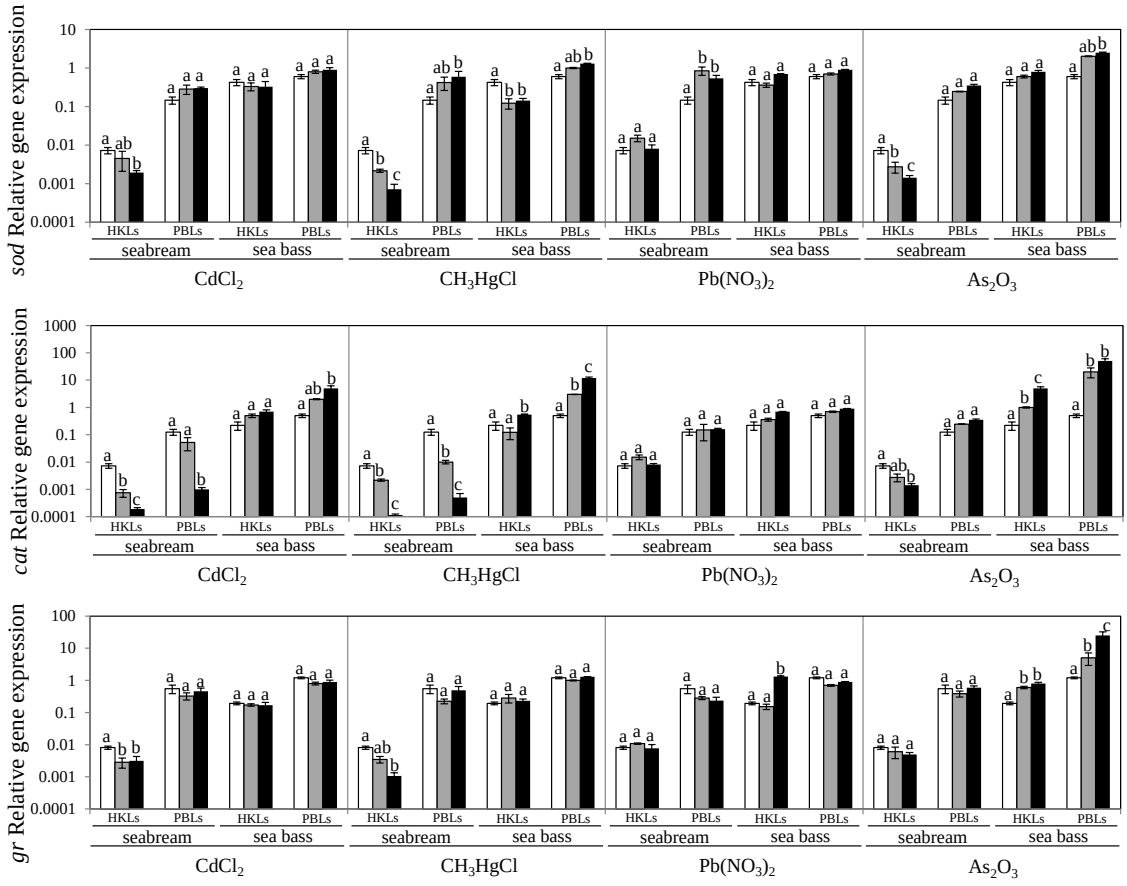

Figure 26. Expression of genes related to oxidative stress (*sod, cat, gr*) in gilthead seabream or European sea bass leucocytes unexposed (white bars) or exposed to the EC_0 (grey bars) or EC_{50} (black bars) of each metal for 24 h. Bars represent the mean ± SEM (n = 4) gene expression relative to the expression of the endogenous control *ef1a* gene. Different letters denote statistically significant differences ($P<0.05$) between control and metal-exposed leucocytes from the same source.

By contrast, it was significantly up-regulated by MeHg exposure in PBLs from both species, Pb exposure in seabream PBLs and As exposure in sea bass PBLs. The *cat* transcription was again down regulated after Cd or MeHg exposure in seabream HKLs or PBLs, or after As exposure in seabream HKLs whilst in sea bass leucocytes it was significantly up-regulated by exposure to Cd (in PBLs) and MeHg or As (in HKLs and PBLs). Similar results were also observed in the *gr* transcription, showing a down regulation after Cd or MeHg exposure in seabream HKLs and a significant increase after As exposure in sea bass HKLs or PBLs. Interestingly, exposure of leucocytes to either EC_0 or EC_{50} provoked roughly the same profile at transcriptional level than the EC_{50} (Figure 26). In addition, Pb resulted the metal showing less impact in the cellular oxidative stress.

3.3. Cellular protection and stress are differently regulated by metals

We evaluated the cellular protection to metals and stress by the expression of *mta* and (*hsp70*) (Figure 27).

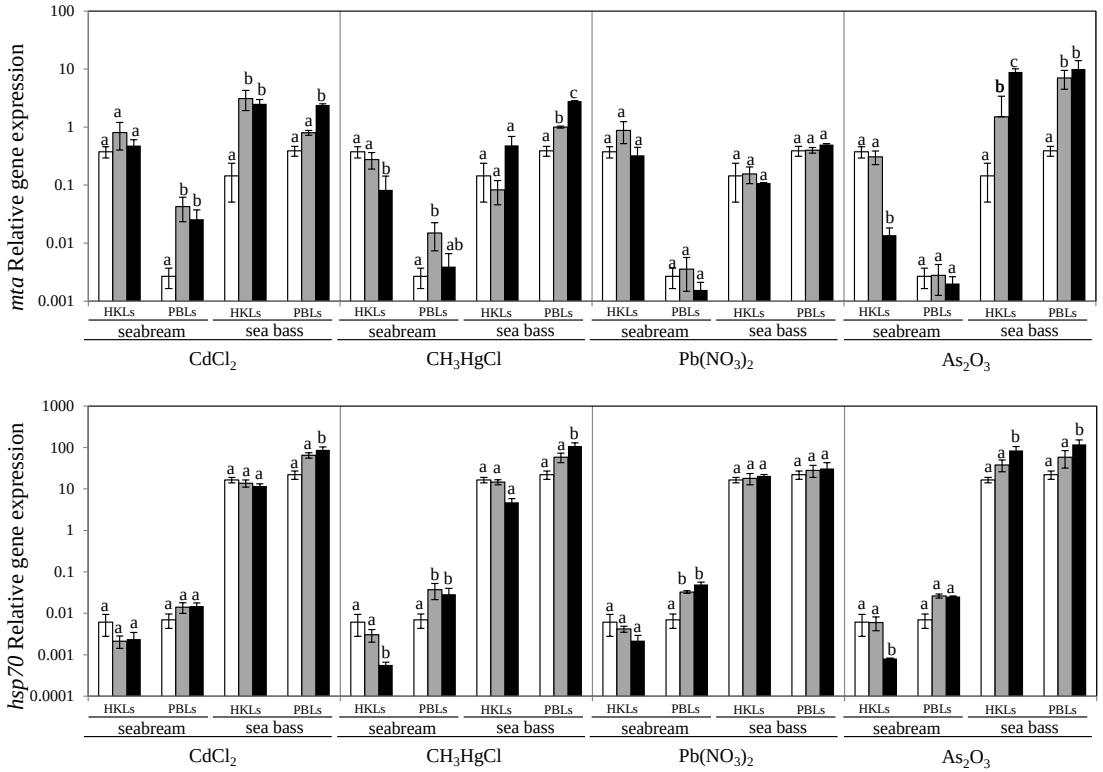

Figure 27. Expression of genes related to cell protection (*mta*) and stress (*hsp70*) in gilthead seabream or European sea bass leucocytes unexposed (white bars) or exposed to the EC_0 (grey bars) or EC_{50} (black bars) of each metal for 24 h. Bars represent the mean ± SEM (n = 4) gene

expression relative to the expression of the endogenous control *ef1a* gene. Different letters denote statistically significant differences ($P<0.05$) between control and metal-exposed leucocytes from the same source.

Surprisingly, *mta* transcription was down regulated after EC_{50} of MeHg or As exposure in seabream HKLs. However, in general, seabream PBLs or sea bass leucocytes exposed to Cd, MeHg and As up-regulated *mta* transcription. On the other hand, Pb exposure does not alter it to a significant level. Regarding *hsp70* transcription, seabream HKLs exposed to EC_{50} of MeHg or As exhibit a significant decrease of the of the *hsp70* mRNA transcription. By contrast, seabream PBLs exposed to MeHg or Pb or seabass PBLs exposed to Cd, MeHg or As enhanced it.

3.4. Metals induce apoptosis cell death

The expression of typical markers for apoptosis (pro-apoptotic Bcl2-associated X gene, *bax*) and caspase 3 (*casp3*) was assayed in fish leucocytes (Figure 28). Leucocytes from seabream and sea bass exposed to EC_0 or EC_{50} of Cd, MeHg or As increased *bax* or *casp3* transcription to a significant extent in most cases, demonstrating the apoptosis cell death process. However Pb only induced *bax* transcription in seabream PBLs.

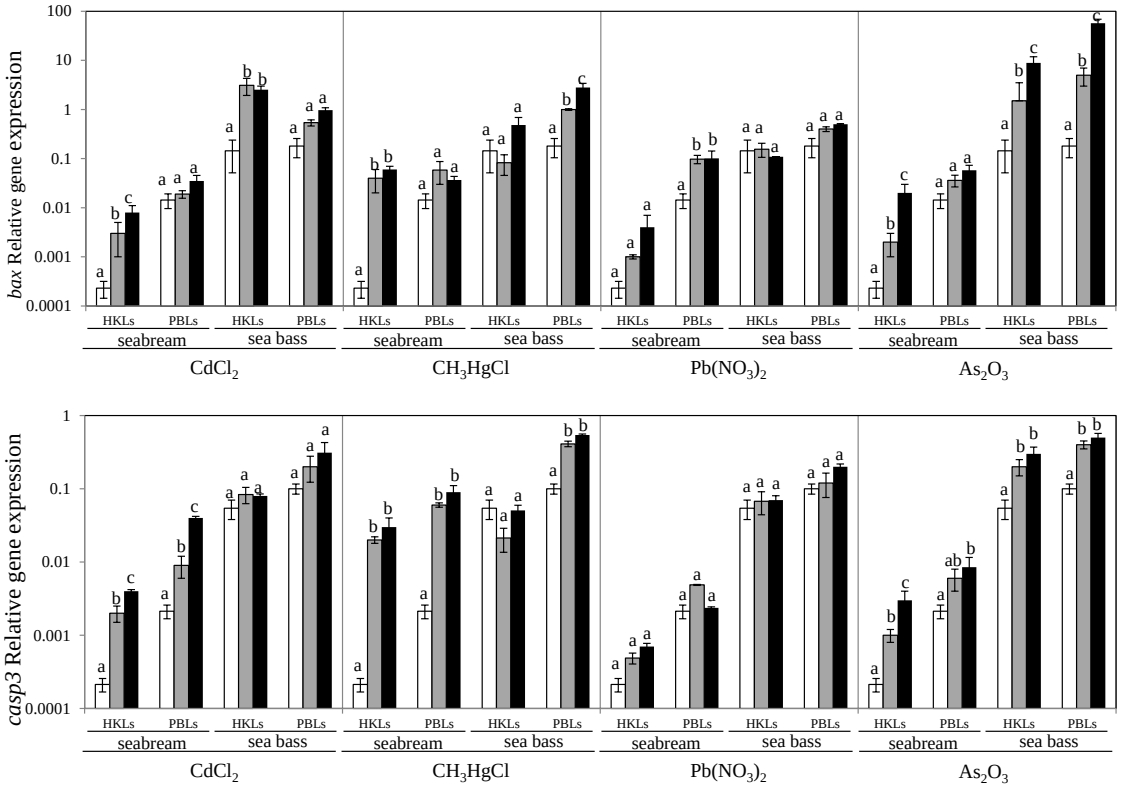

Figure 28. Expression of genes related to cell death (*bax* and *casp3*) in gilthead seabream or European sea bass leucocytes unexposed (white bars) or exposed to the EC_0 (grey bars) or EC_{50} (black bars) of each metal for 24 h. Bars represent the mean ± SEM (n = 4) gene expression relative to the expression of the endogenous control *ef1a* gene. Different letters denote statistically significant differences ($P<0.05$) between control and metal-exposed leucocytes from the same source.

3.5. Metals impaired the expression of immune-related genes

After 24 h exposure to EC_0 or EC_{50} of metals, we have evaluated the expression of selected genes related to the innate and adaptive immunity by real-time PCR in leucocytes from seabream and sea bass (Figure 29). Regarding the *ighm* transcription, it was significantly down-regulated by exposure to EC_0 or EC_{50} of Cd, MeHg and As in both sources of seabream leucocytes and similarly down-regulated by exposure to Pb in sea bass PBLs. The expression of *il1b* was significantly reduced by exposure to all metals in seabream HKLs and PBLs whilst it was slightly modified in sea bass.

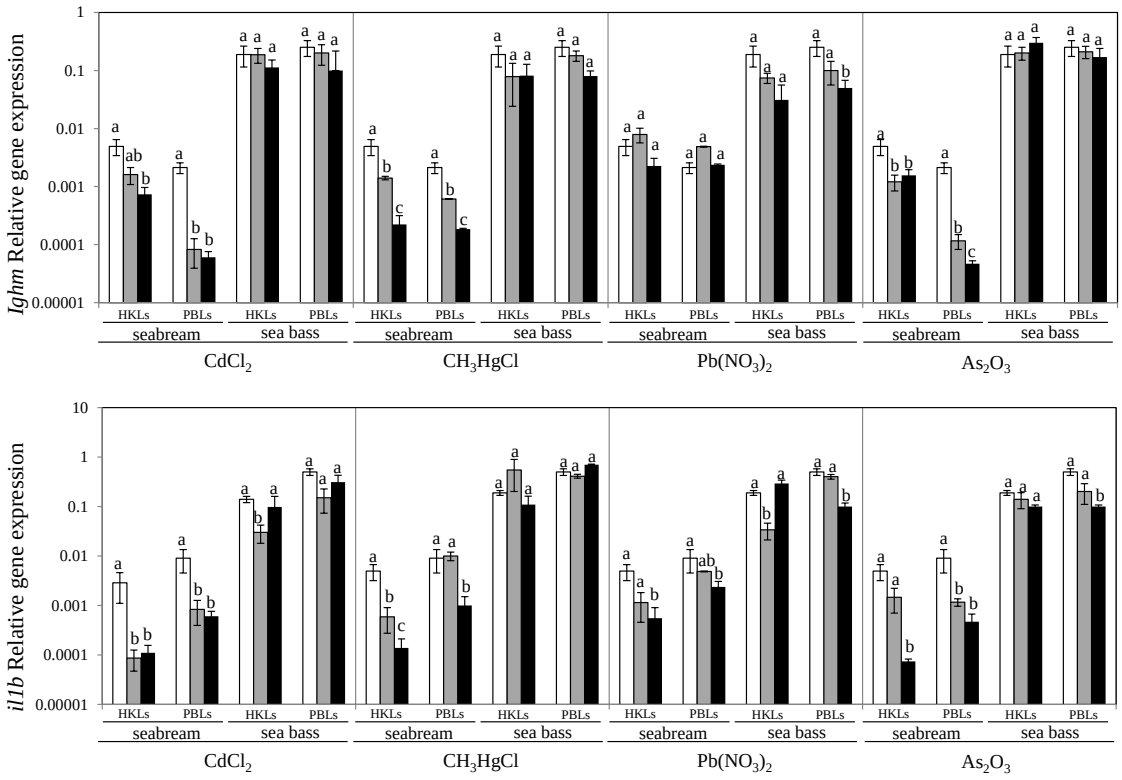

Figure 29. Expression of immune-related genes (*ighm* and *il1b*) in gilthead seabream or European sea bass leucocytes unexposed (white bars) or exposed to the EC_0 (grey bars) or EC_{50}

(black bars) of each metal for 24 h. Bars represent the mean ± SEM (n = 4) gene expression relative to the expression of the endogenous control *efIa* gene. Different letters denote statistically significant differences (P<0.05) between control and metal-exposed leucocytes from the same source.

4. Discussion

In spite of the fact that a large number of environmental chemicals can impair components of the immune system (Bado-Nilles et al., 2009), only few studies have used immune parameters or molecules for environmental risk assessment purposes (Jolly et al., 2012). The deployment of an environmental immunotoxicology approach for aquatic ecosystem biomonitoring is challenging while some functional immune cellular responses should be studied in living cells from fresh tissues. In the present work, we aimed to evaluate the cytotoxicity and alterations at transcriptional level caused by 24 h *in vitro* exposure to metals on head-kidney and blood leucocytes of gilthead seabream and sea bass due to the lack of toxicological studies *in vitro* in fish and the interest might have in the field of Toxicology. Prior to this study, seabream and sea bass HKLs exposure to metals up to 2 h confirmed that these metals provoke leucocyte death, oxidative stress and negatively affect the innate cellular immune functions (Morcillo et al., 2015a, b). We have evaluated the effects at gene level of two different concentrations, EC_0 (sublethal) and EC_{50}. Interestingly, our data show very little differences at gene expression level after metal exposure as we have already documented in the seabream SAF-1 cell line exposed to EC_0 and EC_{50} (Morcillo et al., 2016) and in seabream or sea bass HKLs exposed to low and high metal dosages (Morcillo et al., 2015a, b). These findings have not been described or demonstrated before in fish and explanations for this should be further evaluated.

One of the mechanisms by which metals affect the immune system is through effects on cell viability and proliferation (Hemdan et al., 2005). To achieve the first objective, dose-response experiments were conducted to assess the potential of metals to kill HKLs and PBLs in *vitro*. Though most of the cytotoxicity studies use MTT or NR colorimetric assays to assess cell viability, we opted to analyse leucocyte viability by PI uptake and flow cytometry as in earlier studies (Morcillo et al., 2015a, b). This method is preferred since we use heterogeneous populations (lymphocytes, monocyte-macrophages and granulocytes) containing different amounts of mitochondria and

lysosomes, which could affect to the MTT and NR assays. The cytotoxicity assay revealed dose-dependent curves after 24 h exposure to the metals in leucocytes from gilthead seabream and European sea bass. The EC_{50} values showed that toxicity after 24 h exposure to metals is MeHg>Cd>As>Pb for leucocytes from both species and similarly to that found in seabream and sea bass HKLs after 2 h exposure (Morcillo et al., 2015a, b) and in fish cell lines (Segner, 1998). The fact that MeHg was the most toxic metal for leucocytes could be attributed to a rapid and adverse effect of Hg upon membrane integrity (Schurz et al., 2000). Furthermore, HKLs were more sensitive to the metals than PBLs in the same species; and surprisingly, if we compare between the two species, leucocytes from seabream were more sensitive to the metals than sea bass leucocytes, showing lower EC_{50} values. A potential explanation of this could be due to the fact that head-kidney is composed of leucocytes at all developmental stages (Press et al., 1994) whilst blood leucocytes are mature cells. Interestingly, seabream leucocytes showed lower transcription of genes of the antioxidant system (*sod*, *cat* and *gr*) as well as *hsp70* what could render them more susceptible to ROS induced by metals. In addition, fish exposed to metals showed that the highest bioaccumulation and histopathological damage occurs in the kidney (Fatima et al., 2015; Kessabi et al., 2014) and this could partly explain why leucocytes from this tissue are more sensitive. Further research will confirm or not these hypothesis.

Metal detoxification is an essential process within organisms. Among the mechanisms shown to be important in the cellular protection against metals, and more specific, are MTs (Bourdineaud et al., 2006). In addition, and related to them, HSPs (Morimoto, 2011) and those proteins involved in antioxidant system such as SOD, CAT and GPx are also important. In fact, the MT and ROS levels are related and MTs could also serve as ROS scavengers (Ruttkay-Nedecky et al., 2013). The MTs are the first defense mechanism against the toxicity of metals, mainly Cd, Hg, Cu and Zn, but when metal concentrations reaches high levels and produce protein denaturation, HSPs act to repair the damage (Giudice et al., 1999). However, very few toxicological studies evaluating the impacts of metals on fish leucocyte gene expression are found in the literature. Overall, our data show that seabream HKLs, the most susceptible to metals, exposed to Cd, MeHg or As had down-regulated cellular protection, stress and antioxidant system gene expressions, which partly agrees with the results obtained after 2 h of exposure (Morcillo et al., 2015a). These data indicate that seabream HKLs are not able to

eliminate and compensate the overproduction of ROS induced by metals exposure and therefore an imbalance is generated into the leucocytes. Strikingly, the same genes were mainly up-regulated in seabream PBLs and sea bass leucocytes, which are more resistant to metal cytotoxicity. In Cd-exposed workers, *mta* mRNA levels are also increased in the PBLs (Lu et al., 2001) suggesting the validity of MT expression in leucocytes as a biomarker of metals exposure. Park et al. (2001) reported that the efficacy of MT in protecting against metal-induced lethality was in the order of Cd>>Zn>Cu>Hg, with no protection for Pb, which is confirmed with our results. Regarding *hsp70* gene expression, the results are quite parallel to those obtained for *mta* suggesting their relation and a protective role, at least in sea bass PBLS, which agrees with data obtained after exposure to 10^{-5} M of Cd in sea bass PBLs (Vazzana et al., 2014). Surprisingly, the increase of *sod* (after MeHg or As exposure), *cat* (after Cd, MeHg or As exposure) and to some extent *gr* (after As exposure) expression in sea bass PBLs show a potent mechanism to scavenge ROS and could be one of the reasons that could explain why sea bass PBLs are the most resistant leucocytes tested. Although we did not measure the particular enzyme activities, the gene expression profiles of *sod*, *cat*, *gr*, *mta* and *hsp70* partly support the functional data about the ROS production as observed in seabream and sea bass HKLs (Morcillo et al., 2015a, b). Thereby, the relation between ROS production and the oxidative stress mechanisms and cell protection deserves further investigation in fish leucocytes.

One of the consequences of this ROS overproduction, i.e. triggered by metals, is the induction of apoptosis cell death (Rana, 2008). Thus, based on our data at gene level pointing to this ROS imbalance we have evaluated *bax* and *casp3* gene expression in fish leucocytes. Although we failed to find significant relation between leucocyte transcription of *bax* or *casp3* and the apoptosis process in seabream HKLs after 2 h exposure to metals (Morcillo et al., 2015a), we observed the highest up-regulation in the expression of these genes in seabream HKLs after 24 h of exposure to all metals. On the other hand, sea bass leucocytes and mainly PBLs showed lower up-regulation of *bax* and *casp3* transcription. Therefore, it seems to be a positive relation between the overproduction of ROS inferred and the transcription of apoptosis markers. Our data are in accordance with previous studies in fish which have confirmed that the fish cell death after metal exposure is mainly by apoptosis (Choi et al., 2010; Luzio et al., 2013; Morcillo et al., 2015a, b, 2016; Risso-De Faverney et al., 2004; Sandrini et al., 2009;

Zheng et al., 2014). However, the expression of these apoptosis-related genes does not completely fulfill the observations. Consequently, it is tempting to speculate that other regulators of apoptosis are involved or the differential balance or localization of such proteins might be operating.

Finally, in order to cast some light on the mechanisms leading to the immunotoxicology of metals we evaluated the expression of some immune-related genes. Surprisingly, in seabream leucocytes, and mainly in PBLs, the expression of the B lymphocyte marker, *ighm* gene, was down-regulated after Cd, MeHg and As whilst only in sea bass PBLs exposed to Pb occurred the same. Our data suggest that B lymphocytes, especially in seabream, are altered by metals. In fact, fish lymphocyte *in vitro* proliferation is commonly depressed by Zn, Mn, Hg forms or Cr (Ghanmi et al., 1989; Steinhagen et al., 2004; Voccia et al., 1994). Furthermore, we evaluated the expression of the pro-inflammatory *il1b* gene, expressed in phagocytes (monocyte-macrophages and granulocytes), which suggests that the inflammatory process is sharply inhibited in seabream leucocytes exposed to all metals. However, a slight reduction of this cytokine was observed after metals exposure in sea bass leucocytes. By contrast, *il1b* transcription was up-regulated in rainbow trout head–kidney macrophages exposed to Cu (Teles et al., 2011). Thus, our data about the gene expression of immune-related genes might suggest immunosuppression in the fish leucocytes after 24 h metal exposure, being more significant in seabream leucocytes. Surprisingly, seabream HKLs exposed to Cd and As for 2 h showed up-regulated transcription of *ighm* and *il1b*, respectively whilst Hg forms sharply inhibited them (Morcillo et al., 2015a). On the other hand, only little changes in the expression of immune-related genes were provoked in the European sea bass HKLs exposed to metals (Morcillo et al., 2015b), demonstrating again that this fish species is more resistant to metals. Therefore, the metal and the time-exposure could determinate the activation or inhibition of the immune-related genes and finally the integrity of the fish immune system.

Chapter 5

Toxicological *in vitro* effects of heavy metals on gilthead seabream (*Sparus aurata* L.) head-kidney leucocytes

Abstract

Metals provoke toxicological effects on aquatic animal species, including fish, though their effects on fish leucocytes and immunotoxicology are still limited. In the present work the effects of metals (Cd, Hg, Pb or As) on viability, oxidative stress and innate immune parameters of isolated head–kidney leucocytes from gilthead seabream (*S. aurata*) are studied. Cytotoxicity results indicated that short exposures (30 min or 2 h) to Hg promoted both apoptosis and necrosis cell death of leucocytes whilst Cd, Pb and As did only by apoptosis, in all cases in a concentration- and time-dependent manner. In addition, production of free oxygen radicals was induced by Cd, Hg and As metals. Cd failed to change phagocytosis but Hg and As increased the percentage of phagocytic cells but decreased the number of ingested particles per cell, whilst Pb increased both phagocytic parameters. On the other hand, respiratory burst activity was significantly reduced by incubation with Cd, Hg and As but increased with Pb. Furthermore, the gene expression profiles partly support the functional finding of this work. This study provides an *in vitro* approach for elucidating the metals toxicity, and particularly the immunotoxicity, in fish leucocytes.

1. Introduction

Contamination of aquatic habitats with metals from various industrial and mineral mining sources is a serious environmental problem. The current interest in mineral mining, energy development and use, and dredging will undoubtedly result in further pollution of aquatic environments by such metals as As, Cd, Pb, Hg, and Zn (Sapkota et al., 2008). Metals have been detected in alarming quantities in certain water bodies, particularly at, or near, industrial environments where effluents are routinely discharged. Their bioaccumulation in aquatic organisms and particularly in fish is dangerous not only for their own survival and biology, but also for humans since fish are known to be the greatest inputs of toxic trace elements for humans (EFSA, 2005; Minganti et al., 2010). Thus, considerations about the effects of toxicant exposure on fish biological processes are of great importance for the aquaculture industry management.

Among the adverse effects, metals can produce mortality, alterations in hematological parameters, metabolism, nutrition, reproduction, development and immunodeficiency (Bols et al., 2001; Di Giulio and Hinton, 2008; Sweet and Zelikoff, 2001). Toxicological studies conducted in fish preferentially use field/laboratory *in vivo* exposure because they are more realistic, or fish cell lines, to assess the toxicity whilst the effects on the immune response are still not very well understood. The available results indicated that they largely vary depending on fish (specie, size or tissue), environment (temperature, salinity or photoperiod) and metal (form, route, dosage and exposure time). Thus, *in vivo* studies have usually confirmed that metals decrease the humoral (antibody levels, lysozyme or complement activity, C-reactive protein, etc.) and cellular (leucocyte count, cell- mediated cytotoxic activity, phagocytosis or respiratory burst) immunity of fish, though some other studies have also documented no effect or even increments (Bols et al., 2001; Cuesta et al., 2011). Interestingly, some studies have documented histological alterations of immune tissues connecting with the effects in the leucocyte viability, functions and finally disease resistance (Oliveira-Ribeiro et al., 2002; Guardiola et al., 2013a, b; Mela et al., 2014). Unfortunately, many fewer studies have evaluated the immunotoxicological role of metals *in vitro* on fish leucocytes, which could serve as good models for immunotoxicological studies. To our knowledge, all the information about this respect comes from studies using freshly

isolated leucocytes and none has used the scarce fish immune- or leucocyte-derived cell lines for immunotoxicological purposes. For instance, common carp PBLs exposed to Cr decreased the lymphocyte proliferation, similarly to the HKLs phagocytic functions, at concentrations that did not affect to cell death (Steinhagen et al., 2004). In addition, common carp PBLs exposed to Cd or Zn showed impaired respiratory burst activity, which was unaltered by Pb or Cu (Witeska and Wakulska, 2007) whilst Zn and manganese exposure to carp HKLs induced lymphoproliferation and NK-cell activity (Ghanmi et al., 1989). Red drum PBLs exposed to subtoxic doses of Hg increased cell proliferation whilst toxic doses induced massive calcium flux and activation of tyrosine kinase activity (MacDougal et al., 1996). Interestingly, European sea bass leucocytes reduced the phagocyte functions [respiratory burst measured as production of ROS, phagocytosis or the benefits of macrophage-activating factors (MAF)] by *in vitro* exposure to Hg or Cu (Bennani et al., 1996; Sarmento et al., 2004) whilst Cd-exposure led to their increment (Bennani et al., 1996). Similarly, very few papers have dealt with the toxicological mechanisms caused by metals at gene level. Thus, common carp macrophages exposed to Cu up- regulated the expression of immune-relevant genes [interleukin-1β (*il1b*), il6, tumour necrosis factor-α (*tnfa*), serum amyloid A (*saa*) and trout C-polysaccharide binding protein (*tcpbp*)] (Teles et al., 2011). In European sea bass PBLs, the transcription of heat-shock protein 70 (*hsp70*) was also up-regulated by Cd, but mainly by Cu (Vazzana et al., 2014). Therefore, more studies are needed to clarify the immunotoxicological effects and mechanisms of metals in fish leucocytes. Furthermore, this kind of approximation could be helpful in fish immunotoxicology and should be explored, moreover with the knowledge that *in vitro* cytotoxicity assays with fish cell lines are very well correlated to acute lethality tests *in vivo* (Segner, 2004).

Taking in consideration all the previous data, the aim of this work was to evaluate the immunotoxicological effects of four metals (Cd, As, Pb and Hg) on the head–kidney leucocytes from gilthead seabream (*S. aurata*), the most important farmed fish species in the Mediterranean area. Thus, we exposed seabream HKLs for 30 min or 2 h to ranging concentrations of the cited metals and evaluated the cell death, apoptosis and necrosis pathways, the oxidative stress and phagocyte functions (phagocytosis and respiratory burst) as well as the expression of important genes related to such cellular processes. Results are discussed trying to throw some light in the toxicology of metals in marine fish.

2. Material and methods

2.1. Animals

Thirty specimens of 80±20 g mean weight of the seawater teleost gilthead seabream (*S. aurata*), obtained from a local farm (Murcia, Spain), were kept 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 flow rate of 900 L/h, and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish fed with a commercial pellet diet (Skretting) at a rate of 2% body weight per day. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.2. Head kidney leucocyte isolation

Fish were anaesthetised with benzocaine (4% in acetone) (Sigma) and bled from the caudal vein to avoid excessive tissue contamination with erythrocytes. Head-kidney leucocytes (HKLs) were isolated from each fish under sterile conditions. Briefly, head-kidney was excised, cut into small fragments and transferred to 7 mL of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride, 100 IU/mL penicillin (Life Technologies), 100mg/mL streptomycin (Life Technologies) and 5% foetal bovine serum (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 (1500 rpm, 10 min), counted and adjusted to 10^7 cells/mL in sRPMI. Cell viability was determined by the trypan blue exclusion test.

2.3. Metal exposure

Different salts of the tested metals (Sigma-Aldrich) were used: Cd chloride ($CdCl_2$), trioxide As (As_2O_3), lead (II) nitrate ($Pb(NO_3)_2$), MeHg (II) chloride [CH_3HgCl (MeHg)] and mercury (II) chloride ($HgCl_2$). Each salt was initially dissolved in sterile purified water (Milli-Q) and dilutions for each concentration were daily prepared. Pryor to carrying out the assays, the osmolarity of these solutions was measured in an osmometer (Wescor) to avoid effects due to this parameter. For each fish, 180 μL of seabream isolated HKLs were dispensed in triplicate wells of flat-bottomed 96-well plates (Nunc). Then, 20 μL/well of water (controls) or metal solutions, to make final

concentrations of 0.05–5 mM of Cd, 0.005–0.1 mM Hg forms or 2–10 mM As, were added and exposed for short time, 30 min or 2 h, at 25 °C. Selected concentrations were higher and lower than the effective concentration producing the 50% cell death (EC_{50}) obtained after seabream HKL exposure to the same metals for 24 h whilst exposure times were previously tested in fish (Bennani et al., 1996; Vazzana et al., 2014; Witeska and Wakulska, 2007). In all the assays, we used HKLs from 6 independent specimens (not pooled HKLs) and assayed separately (tested in different days) except in the gene expression studies that we used 4 independent fish specimens.

2.4. Determination of leucocyte viability

In order to determine the gilthead seabream HKLs' viability, we evaluated the abundance of leucocytes in apoptosis and necrosis using a flow cytometry technique based on double-fluorescent labelling (Salinas et al., 2007). For this, after exposure to metals, HKLs were washed and incubated with 5 ng/mL FDA (fluorescein diacetate, green fluorescence; Sigma) and 40 μg/mL PI (red fluorescence; Sigma) for 30 min at 22°C. Samples were then acquired and analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 5000 cells, which were acquired at a rate of 300 cells/s. Side scatter (SSC, granularity), forward scatter (FSC, size), green fluorescence (FL1) and red fluorescence (FL2) parameters were computerised. With this method apoptotic (FDA−PI−), necrotic (FDA−PI+) and viable (FDA+PI−) cells were discriminated and analysed.

2.5. Cellular oxidative stress

The production of ROS, as indicator of the oxidative stress, was determined by dihydrorhodamine 123 (DHR; Life Technologies) (Henderson and Chappell, 1993). DHR 123 is able to diffuse cell membranes and, when oxidized by ROS (mainly by hydrogen peroxide) becomes green fluorescent rhodamine 123, which is sequestered into the mitochondria. After exposure to metals, HKLs were washed and resuspended in 200 μL of fresh medium with 5 μM of DHR 123 and incubated for 30 min at 25 °C. Then, the samples were acquired and analysed in a flow cytometer in order to determine the ROS production, as indicated by the percentage of green fluorescent cells and the mean intensity for each treatment.

2.6. Phagocytosis

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HKLs exposed to metals was also studied by flow cytometry (Rodríguez et al., 2003). Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC; Sigma), washed and adjusted to 5×10^7 cells/mL of sRPMI. After exposure to metals, HKLs were washed and 125 µL of labelled-yeast cells were added, mixed, centrifuged (400 g, 5min, 22 °C), resuspended and incubated (22 °C, 30 min). After incubation, samples were place on ice to stop phagocytosis and 400 µL ice-cold PBS was added to each sample. The fluorescence of the extracellular yeast cells was quenched by adding 40 µL ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HKLs were included in each phagocytosis assay. All samples were analysed in a flow cytometer set to analyse the phagocytic cells, showing the highest SSC and FSC values. Phagocytic activity was measured by 2 parameters: phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the phagocytic cell population whilst the phagocytic capacity was the mean fluorescence intensity.

2.7. Respiratory burst activity

After exposure of HKLs with metals, the respiratory burst activity of seabream HK leucocytes was studied by a chemiluminescence method (Bayne and Levy, 1991). Briefly, samples were incubated with 100 mL of Hank's balanced salt solution (HBSS) containing 1 mg/mL phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma). The plate was shaken and luminescence immediately read in a plate reader (BMG Labtech-Fluostar galaxy) for 1 h at 2 min intervals. The kinetic of the reactions was analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.8. Gene expression by real-time PCR

After 2 h of exposure to the minimum and maximum concentration of each metal (0.05 and 5mM for Cd, 2 and 5mM for As, 0.5 and 5mM for Pb and 0.0005 and 0.1mM for Hg forms) the supernatant was aspirated and TRIzol Reagent (Life Technologies) added to the wells in order to extract the total RNA as indicated by the manufacturer. 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) was synthesized from 1 μg of total RNA using the Super- Script III reverse transcriptase (Life Technologies) with an oligo-dT18 primer. In the present study, the expression of genes involved in apoptosis (*casp3*, *bax* and *bcl2*), metal protection (*mta*), oxidative stress (*sod* and *cat*) and immunity (*ighm* and *tcrb*) lymphocyte markers, antiviral response (Myxovirus resistance protein; *mx*), pro- inflammatory cytokines (*il1b*), antimicrobial peptides (beta-defensin; *bd*) and involved in the generation of ROS during the respiratory burst activity (NADPH oxidase subunits Phox22 and Phox40; *phox22* and *phox40*, respectively) has been evaluated by real-time PCR. Briefly, it was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μL of 2 × SYBR Green supermix, 5 μL of primers (0.6 mM each) and 5 μL of cDNA template) were incubated (10 min, 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 (*ef1a*) RNA content in each sample. Gene names follow the accepted nomenclature for zebrafish (https://wiki.zfin.org/). The primers used in the present study are shown in Table 9. In all cases, each PCR was performed with triplicate samples from four independent and separated specimens.

2.9. Statistical analysis

In all cases the samples were carried out in triplicate wells. Cell death data are expressed as mean±standard error mean, SEM (n=6). The rest of data are presented as fold change to the control samples where values higher than 1 indicates increase and lower than 1 decrease of each parameter. We used, in separate, six different fish for cellular oxidative stress, phagocytosis and respiratory burst activities and four for gene expression studies. Data were statistically analysed by two-way analysis of variance (ANOVA; $P < 0.05$) to determine differences be- tween dose and time followed by post-hoc Tukey's test.

Table 9. Primers used for analysis of gene expression by real-time PCR.

Gene name	Gene abbreviation	GenBank Acc. number	Primer sequences (5′→3′)
Elongation factor 1α	*ef1a*	AF184170	CTGTCAAGGAAATCCGTCGT TGACCTGAGCGTTGAAGTTG
B-cell lymphoma 2	*bcl2*	FM145663	TCAGGAGTGATGTCGAGCTG CAGCCAGGTGCTGACATAGA
Bcl-2 associated X protein	*bax*	AM963390	CAACAAGATGGCATCACACC TGAACCCGCTCGTATATGAAA
Caspase-3	*casp3*	EU722334	CTGATCTGGATGGAGGCATT AGTAGTAGCCTGGGGCTGTG
Metallothionein-A	*mta*	X97276	ACAAACTGCTCCTGCACCTC CAGCTAGTGTCGCACGTCTT
Cu/Zn superoxide dismutase	*sod*	AJ937872	CCATGGTAAGAATCATGGCGG CGTGGATCACCATGGTTCTG
Catalase	*cat*	FG264808	TTCCCGTCCTTCATTCACTC CTCCAGAAGTCCCACACCAT
Immunoglobulin M chain	*igmh*	AM493677	CAGCCTCGAGAAGTGGAAAC GAGGTTGACCAGGTTGGTGT
T cell receptor beta chain	*tcrb*	AM261210	AAGTGCATTGCCAGCTTCTT TTGGCGGTCTGACTTCTCTT
Myxovirus resistance proteins	*mx*	FJ490556, FJ490555, FJ652200	AAGAGGAGGACGAGGAGGAG TTCAGGTGCAGCATCAACTC
Interleukin 1 beta	*il1b*	AJ277166	GGGCTGAACAACAGCACTCTC TTAACACTCTCCACCCTCCA
NADPH oxidase, subunit Phox22	*phox22*	FM148169	CATCAAGAATCCCCCTCAGA TGACAGAGATGGGGTTGTCA
NADPH oxidase, subunit Phox40	*phox40*	AM749961	GCGGAGTTGAACCTGAAGAG TCACCTTCTGTGTCGCTGTC
Beta-defensin	*bd*	FM158209	CCCCAGTCTGAGTGGAGTGT AATGAGACACGCAGCACAAG

3. Results

Cytotoxicity of HKLs exposed to $CdCl_2$, $As2O_3$, $Pb(NO_3)_2$,CH_3HgCl (MeHg) and $HgCl_2$ for 24 h resulted in EC_{50} of 0.134, 2.7, 2.8, 0.012 and 0.026 mM, respectively (un published data). Thus, we aimed to evaluate the effects of shorter exposure on leucocyte viability, oxidative stress, immunity and gene expression in order to cast some light into the metal immunotoxicology and their underlying mechanisms.

3.1. Short exposure to metals induces apoptosis cell death

After 30 min or 2 h exposure to metals, mortality of seabream HKLs, in the form of apoptosis or necrosis, increased in a dose- and time- dependent manner compared to the control or unexposed HKLs (Figure 30). Firstly, Cd, Pb or As exposure for 30 min or 2 h significantly increased apoptosis, but not necrotic cell death, of seabream HKLs. Interestingly, seabream HKLs exposed to MeHg showed a very fast cell apoptosis after 30min of incubation, but not after 2 h, followed by an increment of necrotic cells after 2 h whilst $HgCl_2$ failed to induce apoptosis at any exposure time but induced necrosis after 2 h (Figure 30). Therefore, the EC_{50} provoking apoptosis plus necrosis was not reached by Cd, As, Pb or MeHg whilst it was of 0.09 mM for Hg. Therefore, the 2 h toxicity was in the following order: Hg> MeHg > Cd >As > Pb.

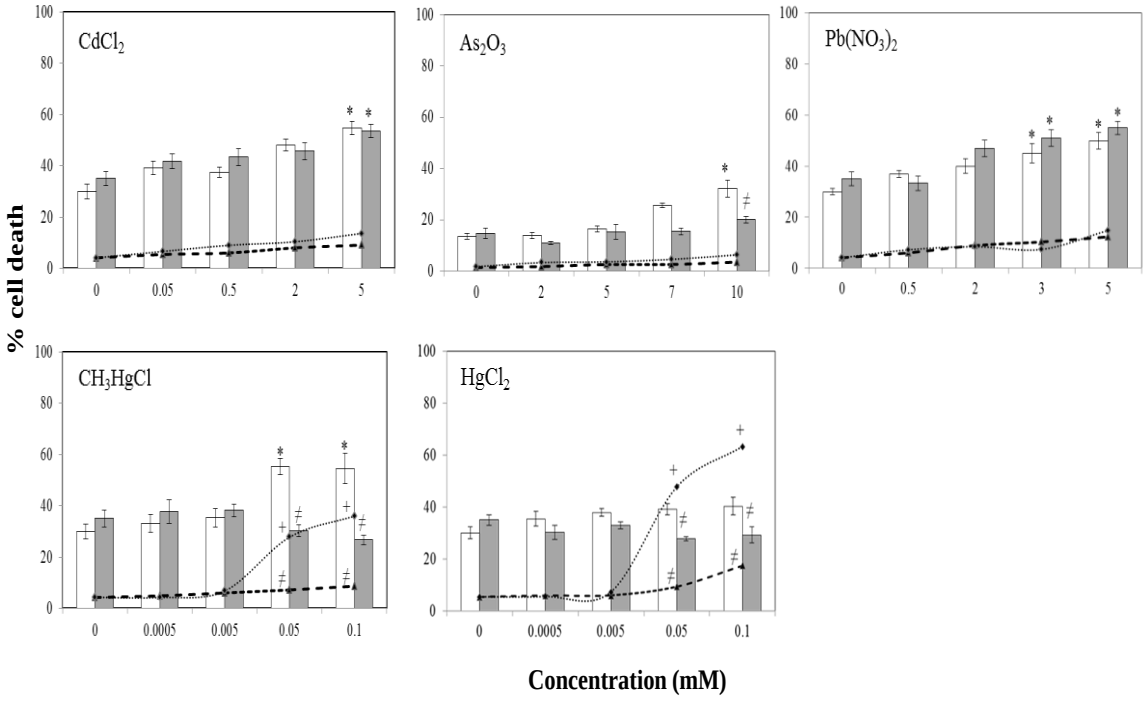

Figure 30. Percentage of cell death via apoptosis (bars) or necrosis (lines) of gilthead seabream head-kidney leucocytes after exposure to different metals for 30 min or 2 h. Apoptosis after 30 min and 2 h is represented by white and black bars, respectively. Necrosis after 30 min and 2 h is represented by discontinuous and dotted lines, respectively. Data represent mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed (apoptosis, *; necrosis, +) or between different times (≠) were denoted.

3.2. Cd, Hg and As induce oxidative stress

The cellular oxidative stress, measured as production of ROS by the DHR 123 reagent, of seabream HKLs exposed to Cd, As or Hg was significantly increased whilst the exposure to Pb failed to change it (Figure 31). In the HKLs exposed to Cd or Hg, the ROS production followed a dose- and time-dependent induction resulting very similar for MeHg and $HgCl_2$. Regarding As, 30 min of exposure failed to affect the oxidative stress but 2 h of exposure produced a significant induction in the ROS production to all the As dosages (Figure 31).

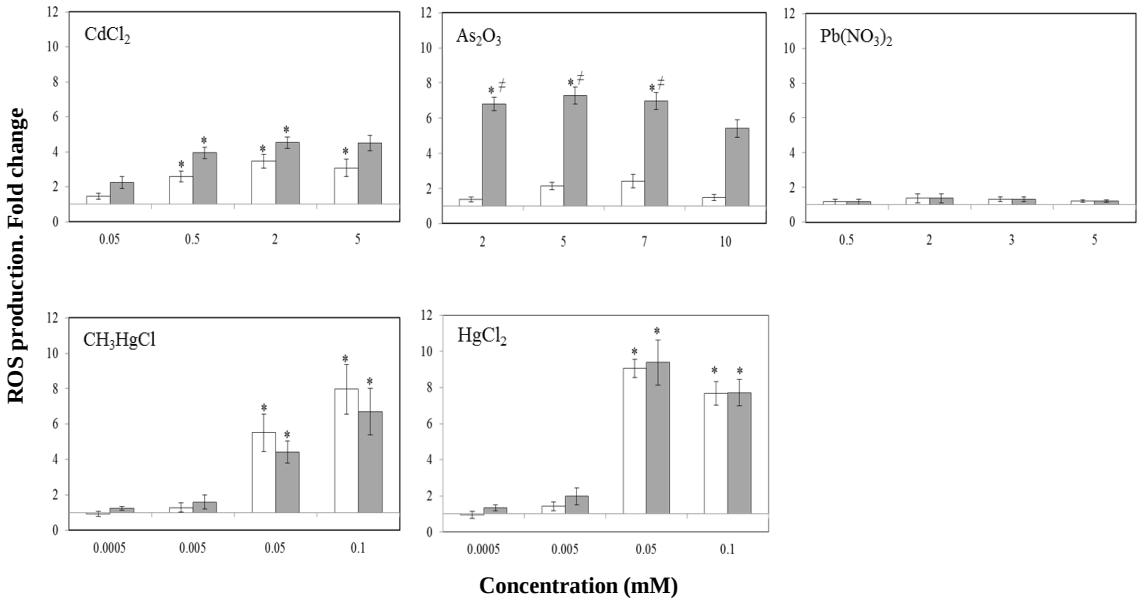

Figure 31. Cellular oxidative stress, measured as the production of ROS, of gilthead seabream head-kidney leucocytes after exposure to different metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change with respect to the control (unexposed leucocytes). Bars represent the mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) or between different times (≠) were denoted.

3.3. Metals alter the expression of genes related to cellular protection, death and oxidative stress

The expression of genes related to metal protection (*mta*), apoptosis (*casp3*, *bax* and *bcl2*) and oxidative stress (*sod* and *cat*) (Figure 32) was evaluated. First, Cd and $HgCl_2$ exposure up-regulated *mta* transcription but MeHg and As down-regulated it to a

significant extent. The *casp3* transcription was altered with respect to control samples but never reached significance. However, *bax/bcl2* ratio was significantly reduced in seabream HKLs exposed to either MeHg or $HgCl_2$, suggesting an anti-apoptotic state and no induction of apoptosis, in sharp contrast to the cell death data described above. Pb, by contrast, increased *bax/bcl2* ratio, which is concomitant with the apoptosis observed. In addition, after demonstration of ROS production in HKLs after metal exposure, the expression of *sod* and *cat* genes, involved in ROS metabolism were determined. Cd, As and Hg metals up-regulated the transcription of *cat* whilst only MeHg did also for *sod* (Figure 32).

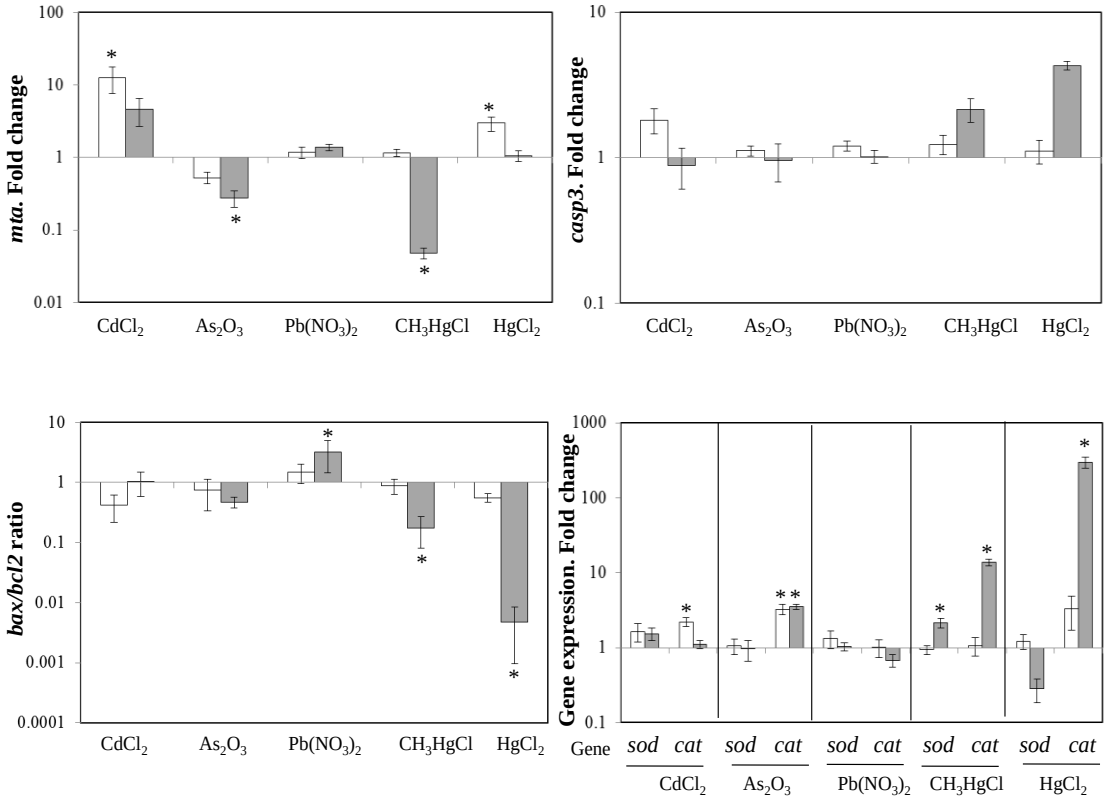

Figure 32. Expression of genes related to cellular metal protection (*mta*), apoptosis (*bax/bcl2* ratio and *casp3*) and oxidative stress (*sod* and *cat*) in gilthead seabream head-kidney leucocytes exposed to the lowest (white bars) and highest (grey bars) metal dosages for 2 h. Data are expressed as gene expression ratio between *bax* and *bcl2*, while the rest are as fold change with respect to the control leucocytes. Bars represent the mean ± SEM (n = 4). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) were denoted.

3.4. Phagocytosis of HKLs is differently affected by Hg, Pb and As

Cd- and As-exposure failed to produce any significant change in the seabream HKLs phagocytic ability though significant differences were observed between HKLs exposed with As for 30 and 2 h (Figure 33). In the case of Hg, the two forms show similar pattern and only high dosages produced a significant increment (Figure 33). Similarly, Pb exposure induced the phagocytic ability in a dose-dependent manner. By contrast, Cd failed to affect the phagocytic capacity of seabream HKLs and As and Hg forms reduced it to a significant level. Moreover, Pb was able, at the lower dosages and exposure times, to increase this phagocytic capacity (Figure 33).

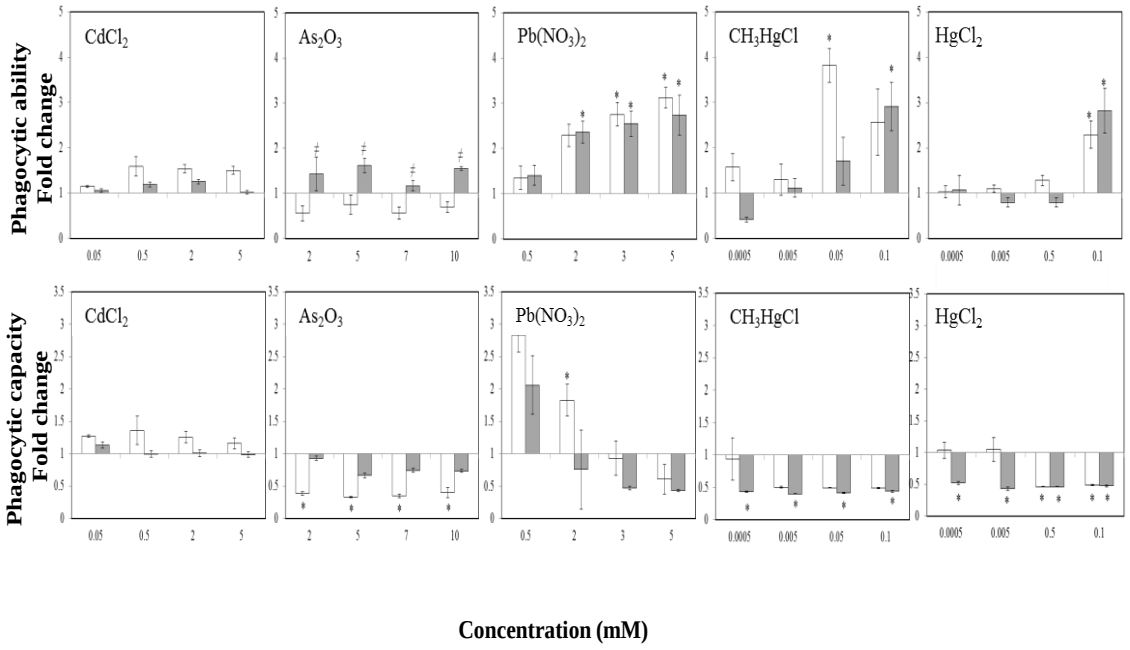

Figure 33. Phagocytic ability and capacity of gilthead seabream head-kidney leucocytes exposed to metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change with respect to the control samples. Bars represent the mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed groups (*) or between different exposure times (≠) were denoted.

3.5. Cd, As and Hg exposure impairs the respiratory burst of seabream leucocytes

The PMA-induced ROS production, the so named respiratory burst activity, of seabream head–kidney leucocytes was significantly impaired to a significant extent after exposure to Cd, As or Hg (Figure 34). Strikingly, Cd and Hg did it in a dose-dependent manner though all the As concentrations produced a very similar effect. However, the respiratory burst was generally increased in seabream leucocytes exposed to Pb though only the highest concentration (5mM) produced a significant increase (Figure 34).

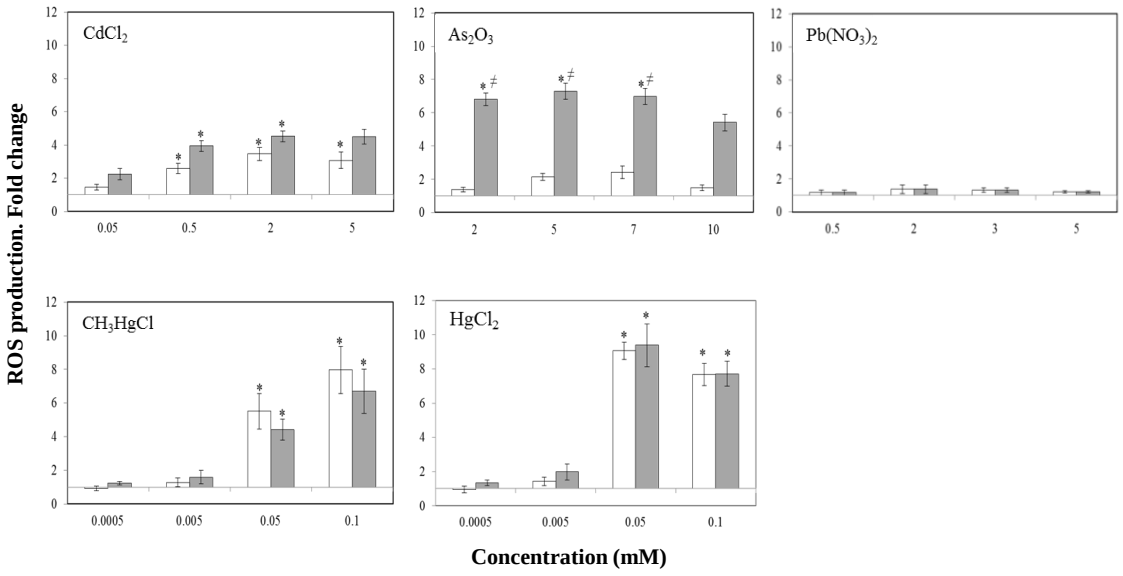

Figure 34. Respiratory burst activity of gilthead seabream head-kidney leucocytes exposed to metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change respect to the control samples. Bars represent the mean ± SEM (n = 6). Asterisks denote statistically significant differences (P<0.05) between control and metal-exposed samples.

3.6. Expression of immune-related genes is altered by Cd, Hg and As

We have evaluated the expression of selected genes related to the innate and adaptive immunity by real-time PCR (Figure 35). Cd exposure indicated an increase in the B lymphocyte marker whilst treatment with $HgCl_2$ reduced both lymphocyte markers and MeHg only did for the marker of T lymphocytes. The expression of the antiviral *mx* gene was significantly up-regulated only by $HgCl_2$ whilst the antimicrobial peptide *bd* did similarly by the two Hg form test (Figure 35). Regarding the *il1b* transcription, it was

significantly up-regulated by the lowest As dosage but similarly down-regulated by the highest dosage of mercurials. Finally, *phox* genes were up-regulated in HKLs exposed to Cd but down-regulated in those exposed to Hg forms. Pb exposure did not alter the expression of any of the tested genes in seabream HKLs.

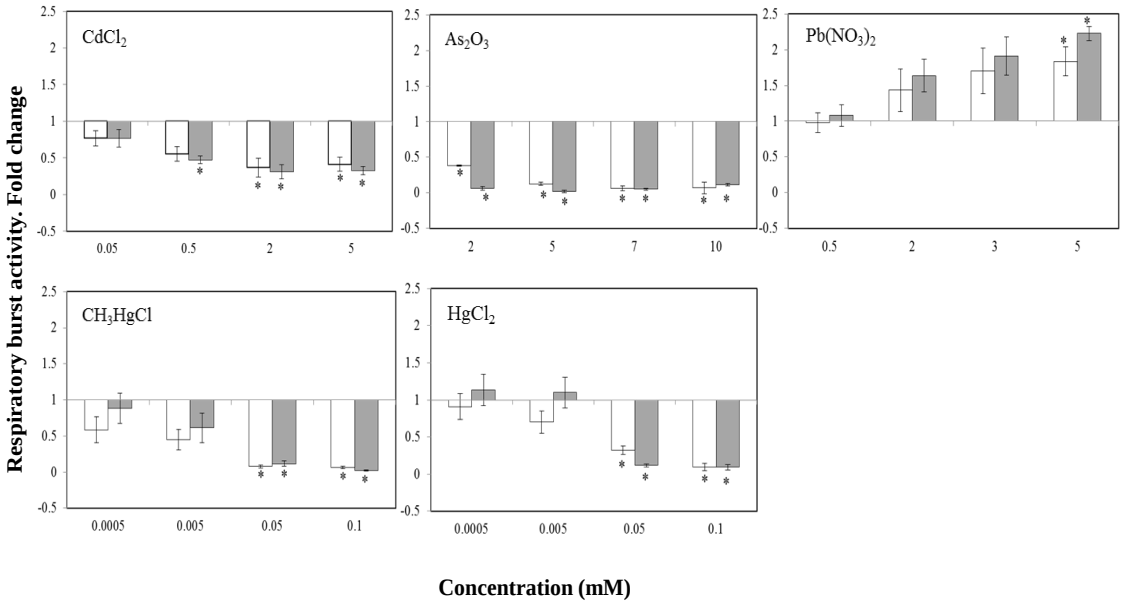

Figure 35. Expression of immune-related genes in gilthead seabream head-kidney leucocytes exposed to the lowest (white bars) and highest (grey bars) metal dosages for 2 h. Data are expressed as gene expression fold change with respect to the control leucocytes. Bars represent the mean ± SEM (n = 4). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) were denoted.

4. Discussion

We have evaluated the effects of metals on viability, oxidative stress and immunity of gilthead seabream HKLs *in vitro* due to the lack of immunotoxicological studies *in vitro* in fish and the interest might have in the field of Toxicology. We have chosen a very short time for leucocyte exposure to metals (30 min and 2 h) since it is known that fish primary cultures of leucocytes show their viability rapidly reduced, mainly in the case of lymphocytes, which in turn are more affected than phagocytes by metals (Witeska and Wakulska, 2007). Thus, previous assays done in fish leucocytes exposed to metals ranged from 10 min to 6 days of exposure (Bennani et al., 1996; Ghanmi et al., 1989;

MacDougal et al., 1996; Sarmento et al., 2004; Steinhagen et al., 2004; Teles et al., 2011; Vazzana et al., 2014; Witeska and Wakulska, 2007). This short time was already enough to show some immunotoxicological signs in fish leucocytes and in our system, the seabream leucocytes. Firstly, we found that cell death, by the sum of apoptosis and necrosis, after 2 h was highest with $HgCl_2$, followed by MeHg, Cd, Pb and As. At 24 h exposure, the toxicity in seabream HKLs was MeHg > HgCl2 > Cd > As > Pb (data not shown) whilst in the seabream SAF-1 cell line was MeHg >As >Cd > $HgCl_2$ > Pb (Morcillo et al., 2016). In addition, our data demonstrate that apoptosis is the main cell death mechanism induced by all the metals tested as previously documented in fish leucocytes (Vazzana et al., 2014) and cell lines (Selvaraj et al., 2013) as well as in mammals (Rana, 2008). Moreover, MeHg induced a rapid apoptosis after 30 min of exposure and after 2 h the cell death detected was only necrosis whilst $HgCl_2$ failed to induce any apoptosis but did the necrosis. This could probably be due to the higher permeability of MeHg provoking therefore a fast and drastic cell death, after only 2 h the cells resulted permeable to the PI labelling and might represent late apoptotic cells more than cells directly killed by a necrosis process. In a similar way, some reports have also documented the induction of apoptosis and necroptosis in fish and mammals (Kim and Sharma, 2004; Krumschnabel et al., 2010; Rana, 2008; Selvaraj et al., 2013). A potential explanation of this could be due to the fact that Hg also initiates lipid peroxidation (Rana, 2008), which can produce alter- ations in cell membrane, and finally, necrosis. We have also evaluated the expression of genes related to the apoptotic pathway: pro-apoptotic *bax*, anti-apoptotic *bcl2* and the executioner *casp3*. Strikingly, we failed to find significant relation of their transcription with the apoptosis measured in the exposed seabream HKLs. Only in the case of Pb we could correlate an increase in the *bax/bcl2* ratio with the observed apoptosis. However, we failed to find a significant up-regulation in the *casp3* transcription in seabream HKLs, as demonstrated in the literature in mammals (Rana, 2008). This could be due to the low apoptosis induced in the seabream leucocytes after only 2 h of exposure. However, we have found that the seabream SAF-1 cell line exposed to Cd, Hg forms and Pb for 24 h showed an increased *bax/bcl2* ratio concomitant with the apoptosis cell death (Morcillo et al., 2016) as well as an increased *casp3* and *casp9* transcription in seabream tissues after waterborne exposure to As (Cordero et al., 2014). This controversy could be due to the implication of other regulators of apoptosis apart from *bcl2* and *bax* genes or to different protein balances or localization.

One of the factors induced by metals leading to cell apoptosis is the overproduction of ROS by the mitochondria. In agreement with the literature, our data demonstrate that seabream HKLs exposed to Hg, Cd and As overproduce ROS and this could also be related to apoptosis cell death (Lushchak, 2011; Rana, 2008). Moreover, As induced the greatest ROS levels in seabream HKLs as also occurred in the seabream SAF-1 (Morcillo et al., 2016) and in PLHC-1 cell lines (Selvaraj et al., 2013). This ROS production has been connected with increased caspase 3 activity and cytochrome C release from the mitochondria, factors that mediate apoptosis though we failed to find such relation in the seabream HKLs, at least in terms of *casp3* transcription and *bax/bcl2* ratios (Rana, 2008). In addition, and surprisingly, we failed to detect any ROS overproduction induced by Pb although it provoked high apoptosis and increments in the *bax/ bcl2* ratio. This suggests that ROS-induced by Pb in seabream HKLs are not responsible for producing cell death. He et al. (2000) demonstrated that Pb produced apoptosis by opening the mitochondrial permeability transition pore (PTP), which didn't open due to oxidative stress. Furthermore, in rats, Pb-induced apoptosis results in an increased *bax/bcl2* ratio (Sharifi et al., 2002), which also agrees with our study.

Regarding the expression of genes related to the antioxidant system, we found that all the metals up-regulated the expression of *cat*, one of the main ROS scavenging enzymes. These data indicate that seabream leucocytes are activated to eliminate and compensate the ROS produced in an effort to balance their levels. These data are, in general, in line with the literature, which show that the antioxidant enzymes, at either gene or protein levels, are good biomarkers of toxicity (Lushchak, 2011; Segner, 1998). Moreover, another important antioxidant enzyme in the protection against metals is the MT (Bourdineaud et al., 2006; Ruttkay-Nedecky et al., 2013). In our study, *mta* transcription was up-regulated by Cd and $HgCl_2$ but down- regulated by MeHg and As. Interestingly, MT, at either gene or protein level, has been found to be increased in fish specimens after metal exposure *in vivo* (García-Santos et al., 2011; Ghedira et al., 2010; Monteiro et al., 2013). *In vitro*, the seabream SAF-1 cells showed up-regulated *mta* transcription when exposed for 24 h to Cd, Hg or Pb, but not to As (Morcillo et al., 2015) and to Cu, Zn and Cd (Minghetti et al., 2011). However, no other study has evaluated the *mta* expression in fish leucocytes after exposure to metals. Our data suggest that *mta* gene expression is not regulated in the very early protection, except after Cd exposure, of fish leucocytes *in vitro* though its functional activity in protection

or antioxidant potential at short exposure times cannot be discarded. Although we did not measure the particular enzyme activities, the gene expression profiles of *sod*, *cat* and *mta* partly support the functional data about the ROS production.

Present results about the effects of metals on fish leucocyte immunity *in vitro* show that seabream HKLs exposed to metals showed differential immune activities: in general, increased phagocytic ability and reduced phagocytic capacity and respiratory burst activities, except Pb that increased all the phagocyte parameters. Cd failed to change the phagocytosis *in vitro* but this activity was increased in gilthead seabream specimens exposed *in vivo* to waterborne As, Cd (Guardiola et al., 2013a, b) or MeHg (Guardiola et al., 2016). Phagocytic activity was unaffected in European sea bass head–kidney macrophages exposed to $HgCl_2$ (Sarmento et al., 2004) and in rainbow trout (*Oncorhynchus mykiss*) leucocytes, from blood or head–kidney, exposed to MeHg or $HgCl_2$, except at those concentration provoking leucocyte death (Voccia et al., 1994). By contrast, phagocytosis activity was decreased by *in vitro* exposure to Cd, Pb, As and Hg in murine macrophages (Christensen et al., 1993; Goering et al., 2000; Kasten-Jolly and Lawrence, 2014; Sengupta and Bishayi, 2002). Unfortunately, the factors leading to such great differences in the effects observed between fish and murine phagocytosis are unknown. A potential explanation could be that seabream phagocytic cells consist mainly of acidophilic granulocytes and they could be modulated in a different way than macrophages. In addition, the increased phagocytic ability of seabream HKLs exposed to Pb, Hg and As could be due to the increase of apoptotic bodies in the samples, which are ingested by phagocytes (De Almeida and Linden, 2005). Likely, some seabream phagocytes are killed by metals whilst others are greatly activated to exert their biological function but these needs to be verified. However, though more phagocytes are activated, they are able to ingest fewer particles, except those exposed to lead. This could be attributed to alteration in the cell-surface receptors mediating the phagocytosis process but other kind of studies should confirm if this is happening.

With the exception of Pb, the rest of metals tested reduced the respiratory burst activity of seabream HKLs, which could suggest that the general immune activity carried out per phagocyte is reduced. In the respiratory burst activity, the production of ROS by granulocytes and macrophages was measured after PMA-activation of the NADH oxidase, radicals that are employed in the phagocytic activity of these cells to kill the pathogens (Babior, 2000). Respiratory burst is a misnomer because it is unrelated to

mitochondrial respiration. In fact, oxidative stress, or the mitochondrial ROS production is not detected by the chemiluminescence technique whilst the PMA-induced ROS production is greatly detected, indicating that this respiratory burst produces much more ROS than the mitochondrial respiration. *In vivo* studies, water- borne exposure to As, Cd or MeHg produced an increase, reduction or no alteration, respectively, in the seabream HKLs respiratory burst (Guardiola et al., 2013a, b). Our data are in line with most of those obtained *in vitro* in which the PMA-stimulated production of ROS was decreased in fish leucocytes exposed to Cr, Cu, Zn or Cd (Bennani et al., 1996; Sarmento et al., 2004; Steinhagen et al., 2004; Witeska and Wakulska, 2007) or in mammalian macrophages exposed to Cd or As (Irato et al., 2001; Palmieri et al., 2007).

In order to cast some light on the immune mechanisms leading to the immunotoxicology of metals, we evaluated the expression of some immune-related genes. Surprisingly, Pb exposure failed to significantly alter any of the studied genes. We firstly evaluated the expression of markers of B and T lymphocytes, *ighm* and *tcrb* genes, respectively because some works have reported that fish lymphocytes are more affected than phagocytes by metals (Witeska and Wakulska, 2007). Our data show increased lymphocyte markers (*ighm*) by Cd suggesting a stimulation of cell proliferation, whilst available data showed that toxic concentrations of Zn, Mn, Hg forms or Cr reduced the proliferation of fish lymphocytes *in vitro* (Ghanmi et al., 1989; Steinhagen et al., 2004; Voccia et al., 1994). However, in the case of Hg forms, our data show a down-regulation in the gene expression of B and T cell markers in agreement with the reduced lymphoproliferation of fish leucocytes (MacDougal et al., 1996; Voccia et al., 1994). We also evaluated the transcription of antiviral and antibacterial effector molecules, Mx and beta-defensin, respectively. Only the *bd* gene was up-regulated by both mercurials and the *mx* only by $HgCl_2$, which suggests that phagocytes are not greatly impaired in their immune functions. Interestingly, we evaluated the expression of pro-inflammatory *il1b* gene, which showed that seabream HKLs were not under an inflammatory process except those exposed to As. This cytokine, among others, was up-regulated by Cu exposure in rainbow trout head-kidney macrophages (Teles et al., 2011). Moreover, we also found up-regulation of seabream HKLs transcription of *phox22* and/or *phox40* by Cd and down-regulation by Hg forms even considering that the respiratory burst was decreased by Cd, Hg forms and As but increased by Pb. The NADPH system is one of the most powerful systems producing

ROS during the respiratory burst. Despite the inhibitory effects of metals in the respiratory burst in fish leucocytes, an increase of *phox* transcription after Cu exposure in rainbow trout macrophages was also evidenced (Teles et al., 2011). Curiously, in mammalian cells, exposure to As or Pb induces the nuclear factor kappa B (NF-κB) activity whilst mercury reduces it (Chen et al., 2001; Woods et al., 2002). This factor is responsible for the activation of cytokine production and many leucocyte functions. Thus, this could partly explain why seabream HKLs exposed to As had increased phagocytic ability and *il1b* transcription. Similarly, seabream HKLs exposed to Pb also resulted in increased phagocyte functions, but not gene expression, pointing also to the activation of the NF-κB. Our data about the gene expression of immune-related genes and phagocyte functions studies failed to show a clear correlation and points to the need for further analysis.

Chapter 6

In vitro immunotoxicological effects of heavy metals on European sea bass (*Dicentrarchus labrax* L.) head-kidney leucocytes

Abstract

The knowledge about the direct effects of metals on fish leucocytes is still limited. We investigate the *in vitro* effects of metals (Cd, Hg, Pb or As) on oxidative stress, viability and innate immune parameters of head-kidney leucocytes (HKLs) from European sea bass (*D. labrax)*. Production of free oxygen radicals was induced by Cd, Hg and As, mainly after 30 min of exposure. Cd and Hg promoted both apoptosis and necrosis cell death while Pb and As did only apoptosis, in all cases in a concentration-dependent manner. Moreover, expression of genes related to oxidative stress and apoptosis was significantly induced by Hg and Pb but down-regulated by As. In addition, the expression of the *mta* gene was up-regulated by Cd and Pb exposure though this transcript, as well as the *hsp70*, was down-regulated by Hg. Cd, MeHg and As reduced the phagocytic ability, whereas $HgCl_2$ and Pb increased it. Interestingly, all the metals decreased the phagocytic capacity (the number of ingested particles per cell). Leucocyte respiratory burst changed depending on the metal exposure, usually in a time- and dose-manner. Interestingly, the expression of immune-related genes was slightly affected by Cd, MeHg, As or Pb being $HgCl_2$ the form producing the greatest alterations, which included down-regulation of immunoglobulin M and hepcidin, as well as the up-regulation of interleukin-1 beta mRNA levels. This study provides an *in vitro* approach for elucidating the metals toxicity, and particularly the immunotoxicity, in fish leucocytes.

1. Introduction

Contamination by metals such as As, Cd, Pb, Hg or Zinc in the marine environment is a severe problem since these pollutants are very persistent. Among the adverse effects, metals can produce mortality, alterations in hematological parameters, metabolism, reproduction, development and immunodeficiency in fish (Bols et al., 2001; Fernández-Trujillo et al., 2011). Regarding this last aspect, increasing attention has been paid to the immune system of fish as an indicator of exposure to environmental pollutants (Zelikoff et al., 1995). Most immunological research involving fish species has been concerned with either their phylogenetic or economic importance; thus, such groups as agnathans, elasmobranchs, and chondrosteans have been studied in attempts to elucidate the evolution of the vertebrate immune system. Also, species such as salmonids (*Oncorhynchus* spp. and *Salmo* spp.), carp (*Cyprinus* spp.) and catfish (*Ictalurus* spp.) have received interest mainly due to their use in aquaculture industries and human consume. Unfortunately, to our days very little is known about the metal effects on fish immunotoxicology and the mechanisms involved.

Traditionally, *in vivo* toxicology tests are followed by mechanistic *in vitro* and/or *ex vivo* tests. However, in recent years *in vitro* tests are gaining traction as alternatives to *in vivo* tests because they are more cost and time effective and have fewer ethical issues (Dobrovolskaia et al., 2009). Therefore, toxicological studies conducted in fish preferentially uses field/laboratory *in vivo* exposure because they are more realistic (Bols et al., 2001; Cuesta et al., 2011) or fish cell lines (Maracine and Segner, 1998) to assess the toxicity while the effects on the immune response are still not very well understood. Thus, *in vivo* exposure to certain metals has been shown to alter innate and acquired immune functions, such as respiratory burst activity, phagocytosis, lymphocyte proliferation or antibody levels as well as interfering with host resistance against infectious pathogens (Cuesta et al., 2011; Zelikoff et al., 1995). Many fewer studies have evaluated the immunotoxicological effects of the toxicants *in vitro* because there are very few available immune-related fish cell lines and the use of freshly isolated leucocytes has attracted little interest among researchers. Strikingly, it has been quite well correlated the cytotoxicity assays in fish cell lines with acute lethality test in fish (Segner, 2004) and it would be interesting to evaluate whether this relation between

the effects of toxicants on isolated leucocytes responses and immunotoxicology *in vivo* could be established in fish.

Focusing on the immunotoxicological effects, it has been demonstrated that *in vitro* exposure of catfish (*Ictalurus melas)* nonspecific cytotoxic cells (NCCs) to different concentrations of soluble Cd inhibited their ability to kill human tumor cells (Viola et al., 1996). Interestingly, European sea bass (*D. labrax)* leucocytes reduced their phagocytosis by *in vitro* exposure to Hg or Cu (Bennani et al., 1996; Sarmento et al., 2004). Similarly, few papers have dealt with their toxicological mechanisms. The toxic effects of pollutants, therefore, often depend on their capacity to produce an oxidative stress response by the increment of mitochondrial levels of ROS in fish (Betoulle et al., 2000; Viarengo et al., 2007) which in turn can then induce the apoptosis cell death (Krumschnabel et al., 2005; Risso-De Faverney et al., 2001). Among the cellular mechanisms to protect from the toxic-induced damage, cells use SOD, CAT, GPx, HSPs and MT, which have also been described in fish. For example, in European sea bass peripheral blood leucocytes (PBLs), *hsp70* transcription was up-regulated at a concentration 10^{-3} M by Cd and mainly by Cu (Vazzana et al., 2014). Similarly, arsenate proved to be the most potent inducer of *hsp70, hsp90* and *mta* transcription in heart cells of common carp (Said et al., 2010). Therefore, more studies are needed to evaluate the *in vitro* immunotoxicological effects of metals, and their mechanisms, in fish leucocytes.

Thus, the present study aimed to throw some light in the toxicology of metals in marine fish leucocytes. We evaluated the immunotoxicological effects of Cd, Hg, Pb and As after 30 min or 2 h exposure, to ranging concentrations of the cited metals. Effects on oxidative stress, cell death (apoptosis and necrosis pathways), as well as phagocyte functions (phagocytosis and respiratory burst) and expression of immune-related genes were studied on the head-kidney leucocytes (HKLs) of European sea bass (*D. labrax*), one of the most important Mediterranean cultured fish species.

2. Material and methods

2.1. Animals

Thirty specimens of 80-100 g body weight of the seawater European sea bass (*D. labrax*), obtained from Culmarex (Murcia, Spain), were kept 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 flow rate of 900 L/h, and 28‰ salinity. The photoperiod was of 12 h light: 12 h dark and fish fed with a commercial pellet diet (Skretting) at a rate of 2% body weight/day. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.2. HKLs isolation

Fish were anaesthetised with benzocaine (4% in acetone) (Sigma) and bled from the caudal vein. HKLs were isolated from each fish under sterile conditions. Briefly, head-kidney was excised, cut into small fragments and transferred to 7 mL of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride, 100 IU/mL penicillin (Life Technologies), 100 mg/mL streptomycin (Life Technologies) and 5% foetal bovine serum (Life Technologies) (Mulero et al., 1998). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 µm), washed twice (400 g, 10 min), counted and adjusted to 10^7 cells/mL in sRPMI.

2.3. Metals exposure

Different salts of the tested metals (Sigma) were used: Cd chloride ($CdCl_2$), MeHg (II) chloride [CH_3HgCl (MeHg)], mercury (II) chloride ($HgCl_2$), lead (II) nitrate ($Pb(NO_3)_2$) and trioxide As (As_2O_3). Each salt was initially dissolved in sterile purified water (Milli-Q) and dilutions for each concentration were daily prepared. Prior to carrying out the assays, the osmolarity of these solutions was measured in an osmometer (Wescor) to avoid effects due to osmolarity. For HKL treatments, 180 µL of freshly isolated European sea bass HKLs from 6 specimens were dispensed in separate wells of flat-bottomed 96-well plates (Nunc). Then, 20 µL/well of water (controls) or metal solutions, to make final concentrations of 0.05-5 mM for Cd, 0.005-0.1 mM for Hg, 0.5-

5 mM for Pb or 2-10 mM for As, were added. All the samples were carried out in triplicate wells. Selected concentrations were higher and lower than the effective concentrations producing the 50% cell death (EC_{50}) obtained after European sea bass HKL exposure to the metals for 24 h of incubation. Cells were exposed for 30 min or 2 h at 25ºC as in previous studies (Bennani et al., 1996; Vazzana et al., 2014; Witeska and Wakulska, 2007).

2.4 Oxidative stress

The production of ROS, as indicator of the oxidative stress, was determined by dihydrorhodamine 123 (DHR 123; Life Technologies) (Henderson and Chappell, 1993). DHR 123 is able to diffuse cell membranes and, when oxidized by ROS (mainly by hydrogen peroxide) becomes to the green fluorescent rhodamine 123, which is sequestered into the mitochondria. After exposure to metals, HKLs were washed and resuspended in 200 µL of fresh medium with 5 µM of DHR 123 and incubated for 30 min at 25ºC. Samples were then acquired and analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10,000 cells, which were acquired at a rate of 300 cells/s. Side scatter (SSC, granularity), forward scatter (FSC, size), green fluorescence (FL1) and red fluorescence (FL2) parameters were computerised. The HKLs production of ROS was indicated by the percentage of green fluorescent leucocytes and the mean intensity for each treatment.

2.5. Leucocyte viability

In order to determine the European sea bass HKLs viability, we evaluated the abundance of leucocytes in apoptosis and necrosis using a flow cytometry technique based on double-fluorescent labelling (Salinas et al., 2007). For this, after exposure to metals, samples were washed and incubated with 5 ng/mL FDA (fluorescein diacetate, green fluorescence; Sigma) and 40 µg/mL PI (red fluorescence; Sigma) for 30 min at 22ºC. Samples were then acquired and analysed in a flow cytometer. With this method apoptotic (FDA^- PI^-), necrotic (FDA^- PI^+) and viable (FDA^+ PI^-) cells were discriminated and analysed.

2.6. Phagocytosis

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by European sea bass HKLs exposed to metals was also studied by flow cytometry (Rodríguez et al., 2003). Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC; Sigma), washed and adjusted to 5×10^7 cells/mL of sRPMI. After exposure to metals, HKLs were washed and 125 µL of labelled-yeast cells added, mixed, centrifuged (400 x g, 5 min, 22ºC), resuspended and incubated at 22ºC for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 µL ice-cold phosphate buffered saline (PBS) was added to each sample. The fluorescence of the extracellular yeast cells was quenched by adding 40 µL ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HKLs were included in each phagocytosis assay. All samples were analyzed in a flow cytometer 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 bacteria (green-FITC fluorescent cells) within the phagocytic cell population whilst the phagocytic capacity was the mean fluorescence intensity.

2.7. Respiratory burst activity

After metal exposure, the respiratory burst activity of European sea bass HK leucocytes was studied by a chemiluminescence method (Chabrillón et al., 2005). Briefly, samples were incubated with 100 mL of Hank's balanced salt solution (HBSS) containing 1 mg/mL phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma). The plate was shaken and luminescence immediately read in a plate reader (BMG Labtech-Fluostar galaxy) for 1 h at 2 min intervals. The kinetic of the reactions was analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.8. Expression of genes by real-time PCR (RT-PCR)

After 2 h of HKL exposure to the minimum and maximum concentration of each metal (0.05 and 5 mM for Cd, 0.0005 and 0.1 mM for Hg, 0.5 and 5 mM for Pb or 2 and 5 mM for As) the supernatant was aspirated and TRIzol Reagent (Life Technologies) added to the wells in order to extract the total RNA as indicated by the manufacturer. 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) was synthesized from 1 mg of total RNA using the SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer. The expression of the selected genes was analysed by real-time PCR (RT-PCR), which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 µL of 2 × SYBR Green supermix, 5 µL of primers (0.6 mM each) and 5 µL of cDNA template) were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample. Gene names follow the accepted nomenclature for zebrafish (https://wiki.zfin.org/). The primers used in the present study are shown in Table 10. In all cases, each PCR was performed with triplicate samples from four specimens.

2.9. Statistical analysis

Data of the cell death are expressed as mean ± standard error mean, SEM (n = 6). The rest of data are presented as fold change to the control samples where values higher than 1 indicates increase and lower than 1 decrease of each parameter. All data were analysed by two-way ANOVA and a Tukey's post-hoc test to determine differences between groups ($P<0.05$). Normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. A nonparametric Kruskal–Wallis test was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPPS 20.0 software (SPSS).

3. Results

Cytotoxicity of HKLs exposed to Cd, MeHg, Hg, Pb and As for 24 h resulted in EC_{50} of 1.5, 0.3, 0.675, 3.2 and 3.4 mM, respectively (unpublished data). Thus, we aimed to evaluate the effects of shorter exposure on leucocyte viability, oxidative stress, immunity and gene expression in order to throw some light into the metal immunotoxicology and their mechanisms. The results of the control or unexposed head-kidney leucocytes regarding the cell activities and relative gene expression are shown as supplementary data (Tables SI and S2).

Table SI. Values of the controls of each activity measured in European sea bass head-kidney leucocytes. Data are expressed ad mean ± SEM (n=6). a.u., arbitrary units.

Activity	Units	Mean±SEM
Cellular oxidative stress (ROS)	Mean fluorescence intensity (a.u.)	277 ± 27.8
Phagocytic ability	Percentage %	35.7 ± 5.27
Phagocytic capacity	Mean fluorescence intensity (a.u.)	21 ± 2.01
Respiratory burst	Maximum slope per minute (a.u.)	60.59 ± 8.22

Table S2. Values of the controls of each gene expression measured in European sea bass head-kidney leucocytes. Data are expressed ad mean ± SEM (n=4).

Gene	**Relative gene expression (*gene* / *ef1a*)**
Catalase (*cat*)	0.02 ± 0.005
Cu/Zn superoxide dismutase (*sod*)	0.03 ± 0.004
Caspase 3 (*casp3)*	0.006 ± 0.0006
Bcl-2 associated X protein *(bax)*	0.05 ± 0.004
Heat shock protein 70 *(hsp70)*	2.36 ± 0.57
Metallothionein A *(mta)*	0.15 ± 0.05
Heavy chain of immunoglobulin M *(ighm)*	0.019 ± 0.006
T-cell receptor beta chain *(tcrb)*	0.02 ± 0.006
Interferon-induced GTP-binding protein Mx *(mx)*	0.0008 ± 0.0001
Interleukin-1beta *(il1b)*	0.19 ± 0.08
NADPH oxidase subunit p40phox *(phox40)*	0.013 ± 0.003
Hepcidin *(hepc)*	0.0009 ± 0.0003

Table 10. Primers used for analysis of gene expression by real-time PCR in European sea bass.

Gene name	Gene abbreviation	Acc. number	Sequence (5'-3')
Elongation factor 1-alpha	*ef1a*	AJ866727	CGTTGGCTTCAACATCAAGA
			GAAGTTGTCTGCTCCCTTGG
Catalase	*cat*	FJ860003	GAGGTTTGCCTGATGGCTAC
			TGCAGTAGAAACGCTCACA
Cu/Zn superoxide dismutase	*sod*	FJ860004	TGTTGGAGACCTGGGAGATG
			ATTGGGCCTGTGAGAGTGAG
Caspase 3	*casp3*	DQ345773	AATTCACCAGGCTTCAATGC
			CTACGGCAGAGACGACATA
Bcl-2 associated X protein	*bax*	FM011848	TGTCGACTCGTCATCAAAGC
			CACATGTTCCCGGAGGTAGT
Heat shock protein 70	*hsp70*	AY423555	CTGCTAAGAATGGCCTGGAG
			CTCGTTGCACTTGTCCAGAA
Metallothionein A	*mta*	AF199014	GCACCACCTGCAAGAAGACT
			AGCTGGTGTCGCACGTCT
Heavy chain of immunoglobulin M	*ighm*	FN908858	AGGACAGGACTGCTGCTGTT
			CACCTGCTGTCTGCTGTTGT
T-cell receptor beta chain	*tcrb*	FN687461	GACGGACGAAGCTGCCCA
			TGGCAGCCTGTGTGATCTTCA
Interferon-induced GTP-binding protein Mx	*mx*	AM228977	GTCTGGAGATCGCCTCT
			TCTCCGTGGATCCTGATGGAGA
Interleukin-1beta	*il1b*	AJ269472	CAGGACTCCGGTTTGAACAT
			GTCCATTCAAAAGGGGACAA
NADPH oxidase subunit p40phox	*phox40*	AM490068	GCGGAGTTGAACCTGAAGAG
			TCACCTTCTGTGTCGCTGTC
Hepcidin	*hepc*	DQ131605	CCAGTCACTGAGGTGCAAGA
			GCTGTGACGCTTGTGTCTGT

3.1. Cd, Hg and As induce oxidative stress

The cellular oxidative stress, measured as production of ROS, showed that European sea bass HKLs exposed to Cd or Hg forms were induced to overproduce ROS in a dose-dependent manner being significant only at the highest dosages (Figure 36 and Supplementary data 3.SIA). In sharp contrast, As provoked and increase in the HKLs ROS that was highest with the lowest dosage after 30 min of exposure and the effect was decreasing with the concentration and time (Figure 36 and 3.SIA). However, exposure of sea bass HKLs to Pb did no significantly alter the ROS production.

Interestingly, in the case of Hg forms and As, the highest ROS production was provoked in the HKLs exposed for 30 min and at longer times the effect was abolished.

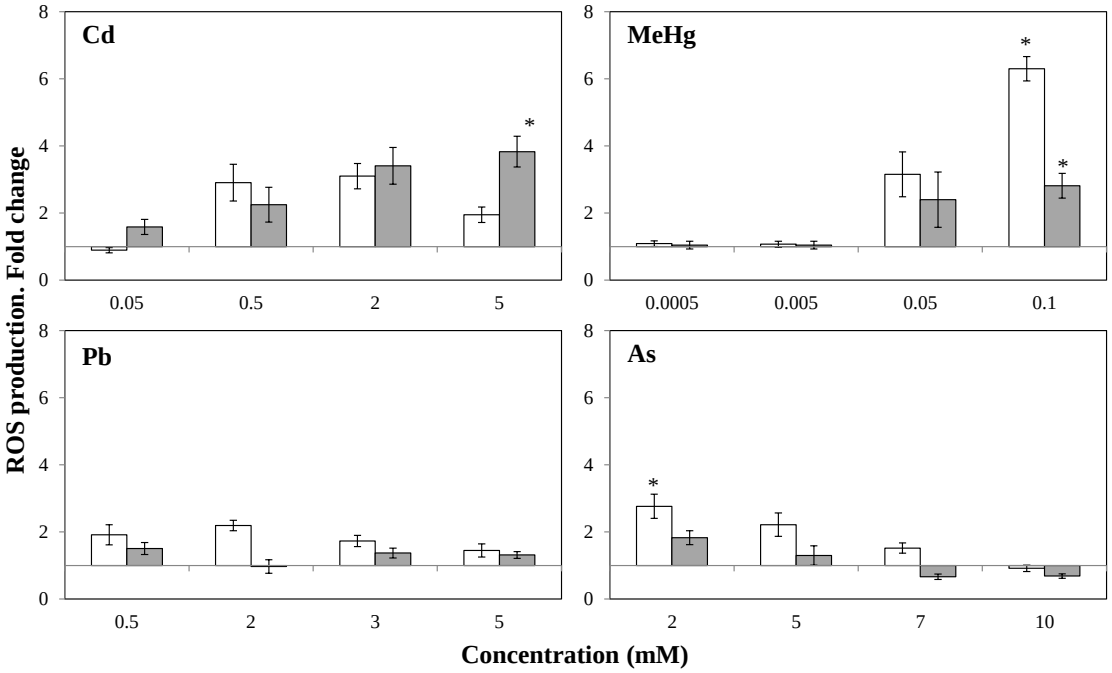

Figure 36. Cellular oxidative stress, measured as the production of ROS, of European sea bass head-kidney leucocytes after exposure to different metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change respect to the control (unexposed leucocytes). Bars represent the mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) or between different times (≠) were denoted.

3.2. Short exposure to all metals induces apoptosis cell death

After 30 min or 2 h exposure to metals, mortality of European sea bass HKLs, in the form of apoptosis and/or necrosis, was increased in a dose- or time-dependent manner compared to the controls (Figure 37 and 3.SIB). Firstly, Pb or As exposure for 30 min or 2 h significantly increased apoptosis, but no necrosis, cell death of European sea bass HKLs. Interestingly, sea bass HKLs exposed to Cd or Hg forms significantly induced cell apoptosis after 30 min of incubation, but not after 2 h, followed by a significant increment of necrotic cells after 2 h, higher in the case of Hg (Figure 37 and 3.SIB).

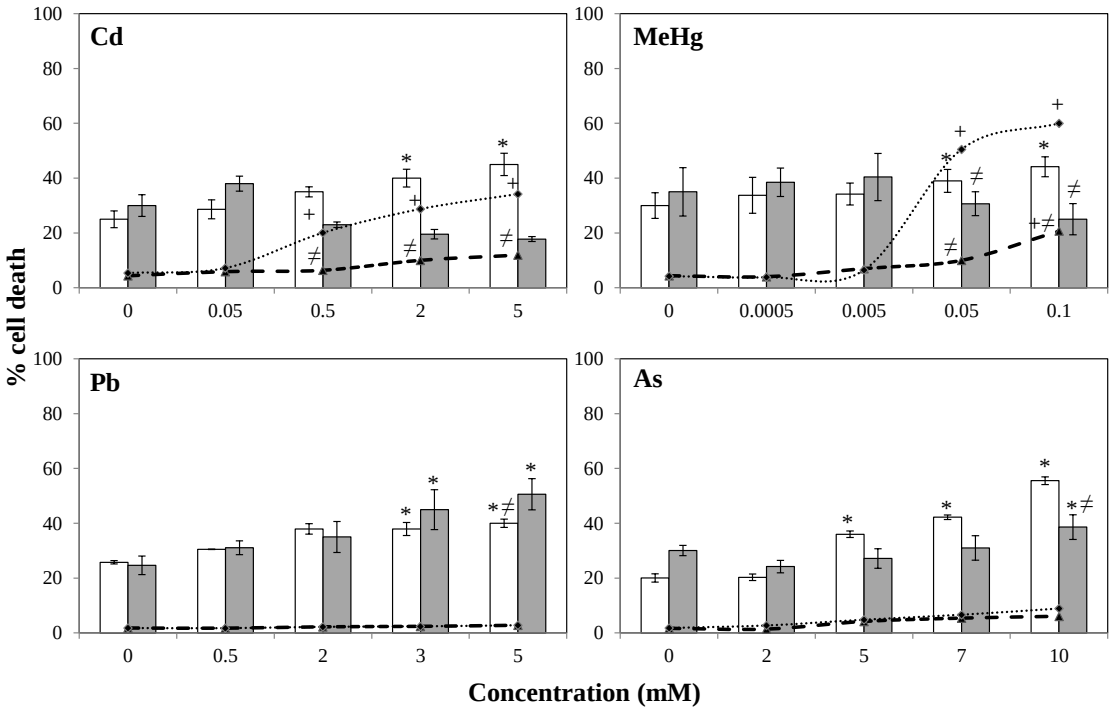

Figure 37. Percentage of cell death via apoptosis (bars) or necrosis (lines) of European sea bass head-kidney leucocytes after exposure to different metals for 30 min or 2 h. Apoptosis after 30 min and 2 h is represented by white and black bars, respectively. Necrosis after 30 min and 2 h is represented by discontinuous and dotted lines, respectively. Data represent mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed (apoptosis, *; necrosis, +) or between different times (≠) were denoted.

3.3. Expression of genes related to cellular oxidative stress, death and protection

Once observed that metals induce ROS production and apoptosis we further evaluated these cellular processes at gene level (Figure 38 and 3.SIC), evaluating their transcription after 2 h of exposure to the lowest and highest dose of each metal (Cd, 0.05 and 5 mM; MeHg, 0.0005 and 0.1 mM; Pb, 0.5 and 2 mM; As, 2 and 10 mM). Firstly, HKL exposure to Hg forms and Pb significantly up-regulated *sod* and *cat* gene expression. By contrast, Cd did to alter them whilst As decreased the *sod* and *cat* transcription, the last one at significant level. Regarding the leucocyte death by apoptosis we evaluated the expression of pro-apoptotic Bcl2-associated X gene (*bax*) and caspase 3 (*casp3*) (Figure 38 and 6.SIC).

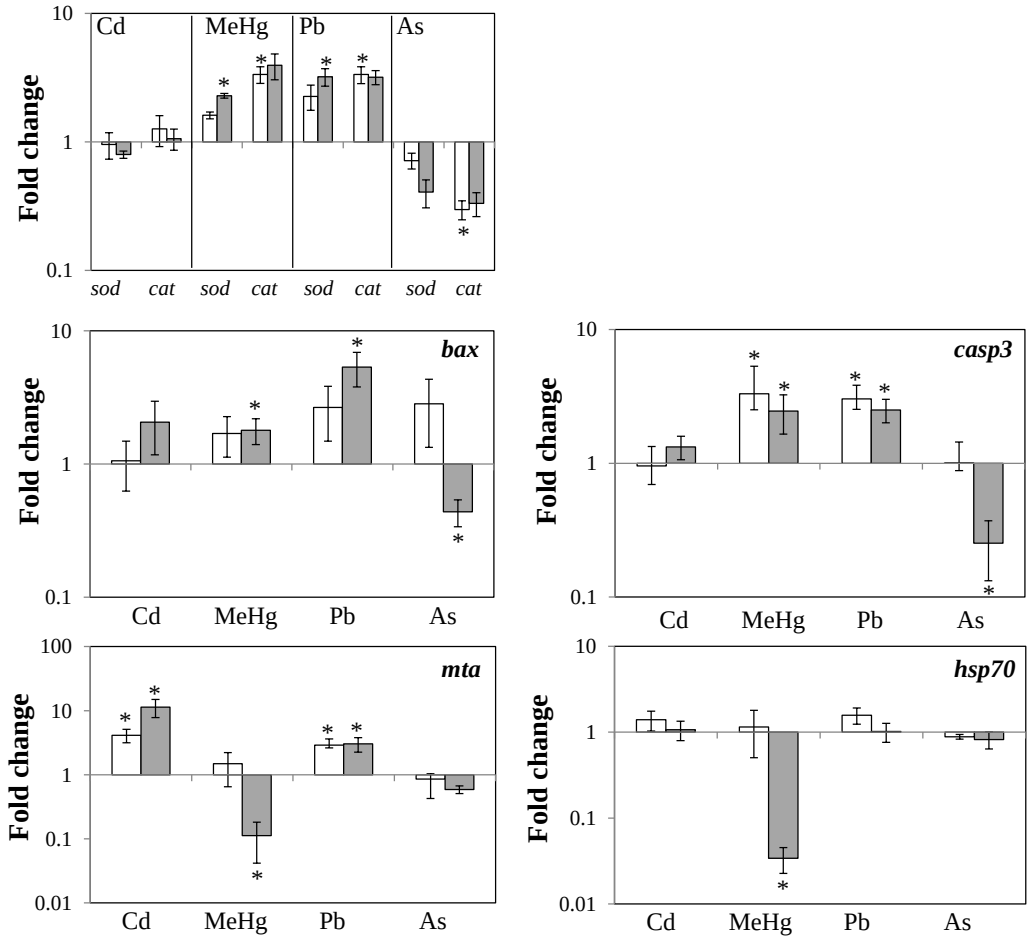

Figure 38. Expression of genes related to cellular apoptosis (*bax* and *casp3*), metal protection (*mta*), stress (*hsp70*) and oxidative stress (*sod* and *cat*) in European sea bass head-kidney leucocytes exposed to metals to the lowest (white bars) and highest (grey bars) metal dosages used in the present work for 2 h. Data are expressed as fold change respect to the control leucocytes. Bars represent the mean ± SEM (n = 4). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) were denoted.

Interestingly, both genes were significantly up-regulated in sea bass HKLs exposed to Hg forms or Pb while down-regulated to a significant extent after exposure to the highest dose of As. Finally, Pb and mainly Cd exposure induced a significant up-regulation of *mta* gene expression; however, Hg forms down-regulated *mta* transcription (Figure 38 and 6.SIC). Moreover, Hg forms induced a strong and significant down-regulation of *hsp70* gene expression but the rest of metals tested failed to regulate its transcription.

3.4. Phagocytic parameters of HKLs were differently affected by metals

Phagocytic activity was determined as the percentage of phagocytic cells, the phagocytic ability (Figure 39 and 6.SID) and the mean fluorescence intensity, the phagocytic capacity (Fig. 40 and 3.SIE). Exposure to Cd, MeHg and As produced a general statistically significant reduction of the HKLs phagocytic ability. By contrast, exposure to $HgCl_2$, and mainly Pb, significantly increased the phagocytic ability of sea bass HKLs. On the other hand, all the metals produced a significant reduction in the phagocytic capacity after 30 min or 2 h of exposure.

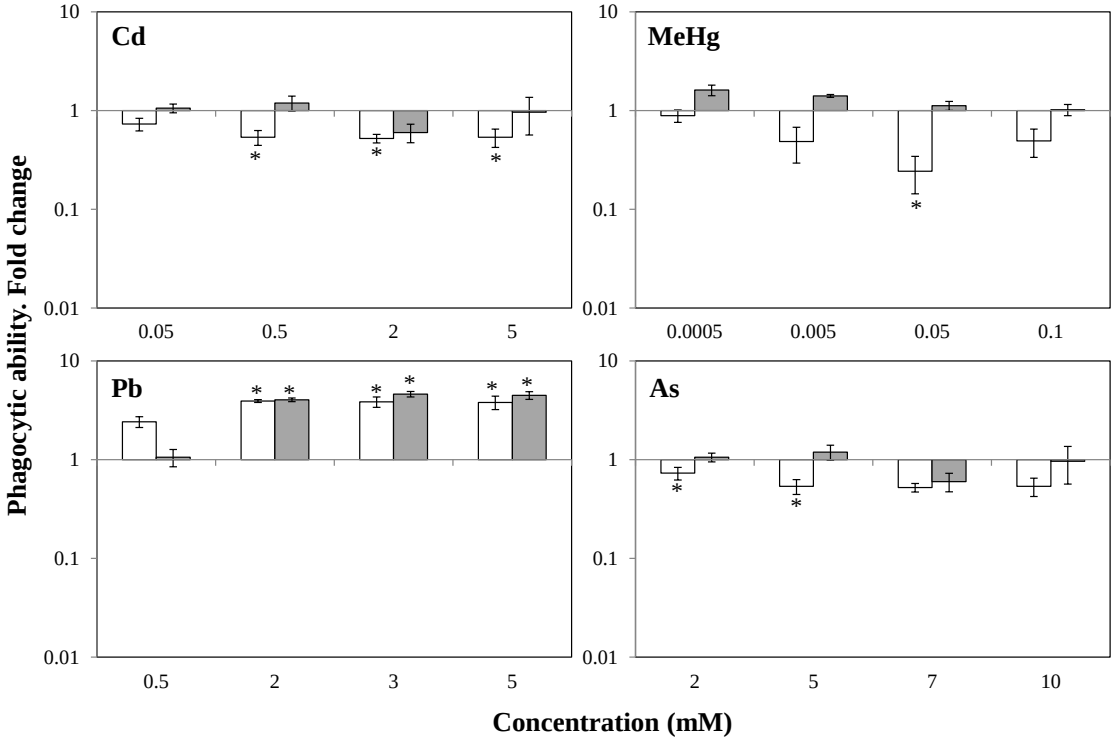

Figure 39. Phagocytic ability of European sea bass head-kidney leucocytes exposed to metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change respect to the control samples. Bars represent the mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed groups (*) or between different exposure times (≠) were denoted.

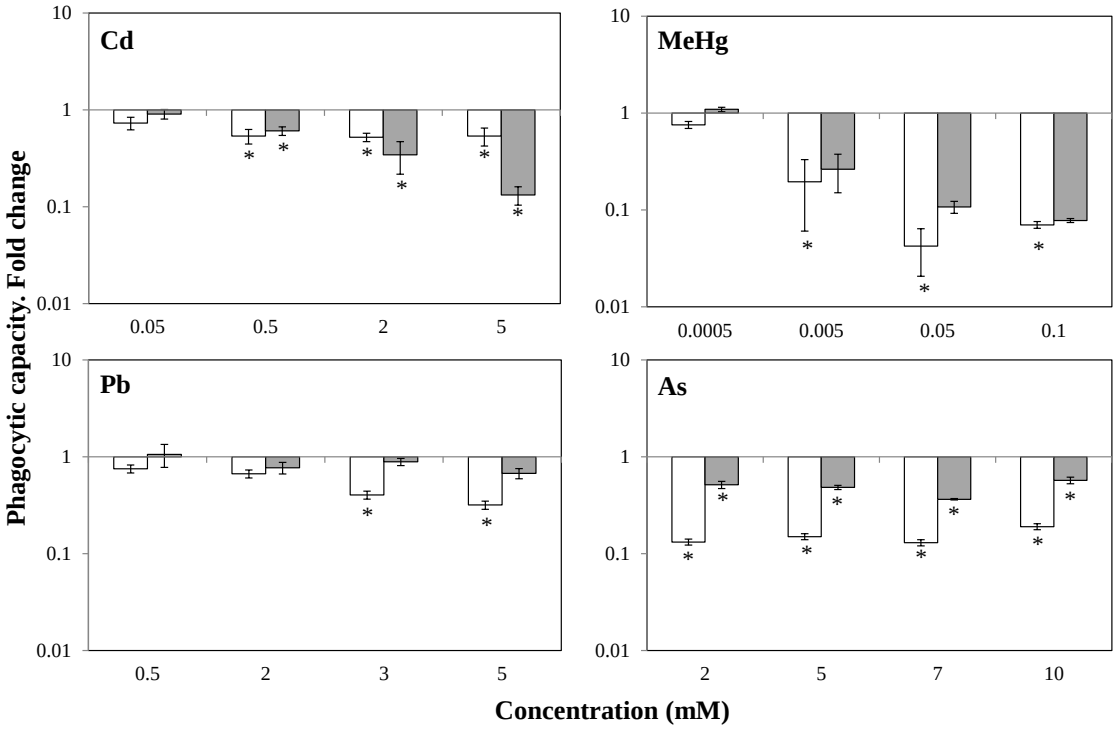

Figure 40. Phagocytic capacity of European sea bass head-kidney leucocytes exposed to metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change respect to the control samples. Bars represent the mean ± SEM (n = 6). Statistically significant differences ($P \leq 0.05$) between control and metal-exposed groups (*) or between different exposure times (≠) were denoted.

3.5. Respiratory burst of HKLs was greatly impaired by As

The PMA-induced ROS production, named respiratory burst, of sea bass HK leucocytes was also determined after exposure to metals (**Figure 4I and 3.SIF**). First, Cd did not change the respiratory burst of sea bass HKLs while As produced the greatest reduction, to a significant extent, in a similar level in HKLs exposed to 5-10 mM. However, Hg forms and Pb produced little changes, some of them significant, which varied from inhibition to increase of the respiratory burst.

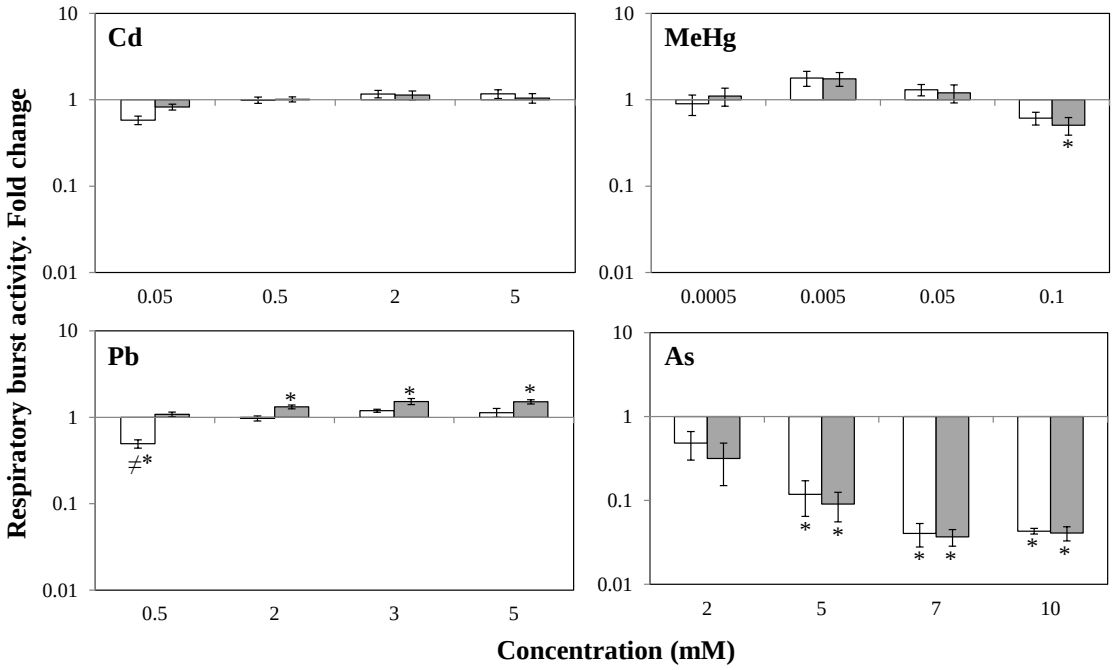

Figure 41. Respiratory burst activity of European sea bass head-kidney leucocytes exposed to metals for 30 min (white bars) or 2 h (grey bars). Results are expressed as fold change respect to the control samples. Bars represent the mean ± SEM (n = 6). Asterisks denote statistically significant differences (P<0.05) between control and metal-exposed samples.

3.6. Metals slightly affected to the expression of immune-related genes

Once observed significant changes in the phagocyte-related innate immune functions caused by metals we tested whether the expression of immune genes was altered. Thus, chain of immunoglobulin M (*ighm*), T-cell receptor beta chain (*tcrb*), interleukin-1 beta (*il1b*), antimicrobial peptide (*hepc*), antiviral (*mx*) and NADPH oxidase subunit p40phox (*phox40*) were analyzed after 2 h exposure of sea bass HKLs to the highest and lowest dosages of each metal (Figure 42 and 3.SIG). Thus, exposure to Cd significantly decreased only the *mx* gene expression while Pb up-regulated the transcription of *phox40*. However, As did not affect the expression of any tested gene. Finally, exposure of sea bass HKLs to MeHg resulted in significant up-regulation of *phox40* and *hepc* transcription (Figure 42). By contrast, $HgCl_2$ up-regulated the *il1b* mRNA levels but also down-regulated those of *ighm* and *hepc* (Figure 3.SIG).

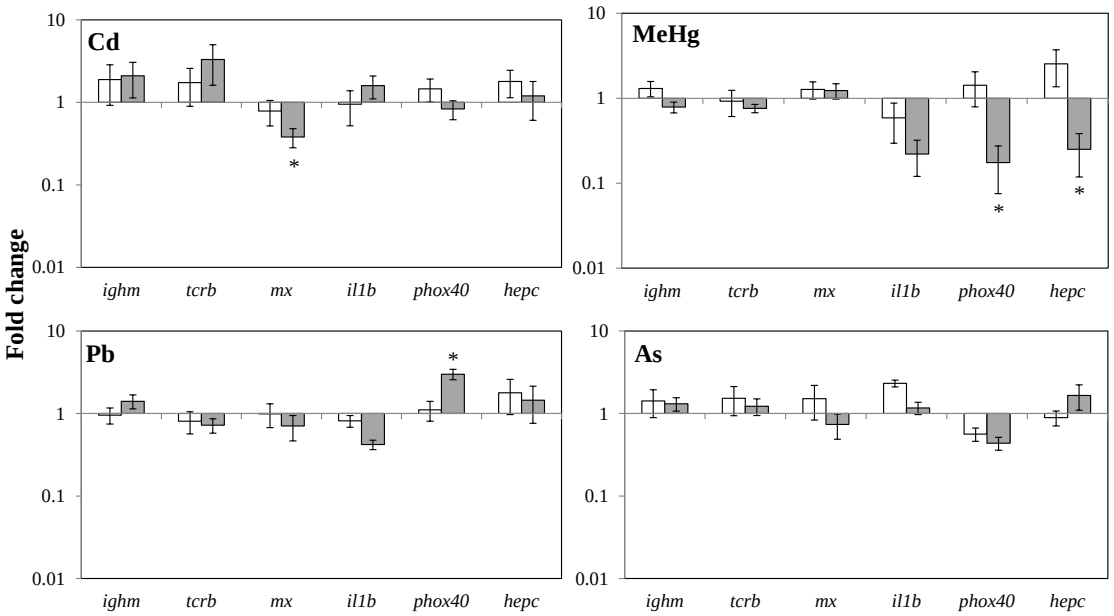

Figure 42. Expression of immune-related genes in European sea bass head-kidney leucocytes exposed to metals to the lowest (white bars) and highest (grey bars) metal dosages for 2 h. Data are expressed as gene expression fold change respect to the control leucocytes. Bars represent the mean ± SEM (n = 6). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) were denoted.

4. Discussion

The use of cell lines to test the toxicity of aquatic pollutants, including metals, is a valuable alternative to fish bioassays. However, sometimes, fish cell lines might not be as good models for toxicological studies and then the use of *in vitro* explants or primary cultures may lead to a significant advance in our knowledge of the toxicology and its mechanisms. This is the case in the field of fish immunotoxicology, in which very few cell lines derived from immune tissues are available and they have never been applied to this end. Thus, some papers have evaluated the immunotoxicology of metals using fish leucocytes *in vitro* (Bennani et al., 1996; Ghanmi et al., 1989; Sarmento et al., 2004; Steinhagen et al., 2004; Teles et al., 2011; Witeska and Wakulska, 2007). With the aim to bring some light in this field, we evaluated the immunotoxicological effect of metals using freshly isolated head-kidney leucocytes of European sea bass, considered one of the most important Mediterranean cultured fish species.

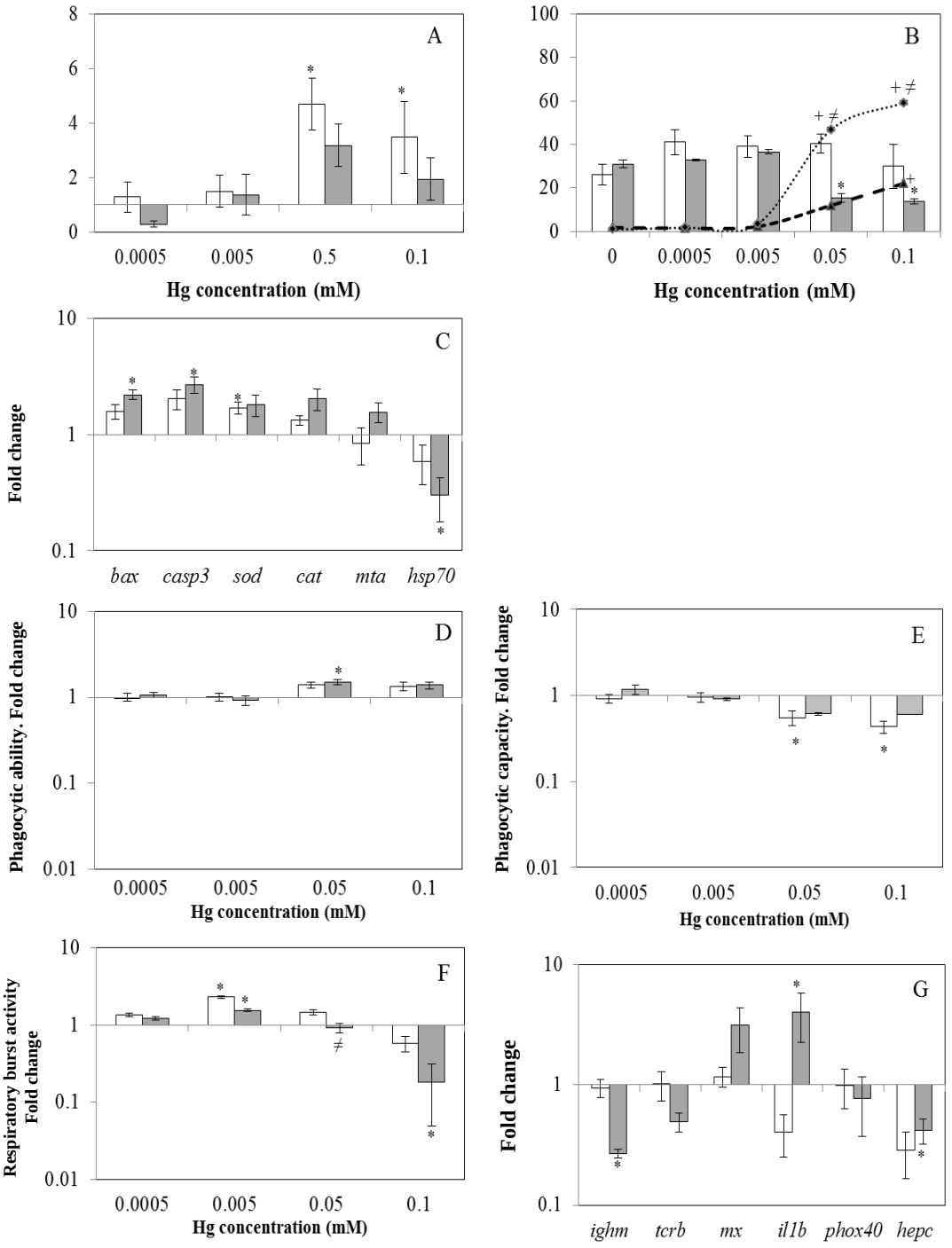

Figure 3.SI. Effects of $HgCl_2$ on European sea bass head-kidney leucocytes oxidative stress, viability, immunity and gene expression. Legend as in Figs.1 to 7. Data in A, B, D, E and F correspond to 30 min (white bars) and 2 h (grey bars) metal exposure and are presented as mean ± SEM (n = 6). Data in C and G correspond to the lowest (white bars) and highest (grey bars) metal dosages used in the present work for 2 h. Data are expressed as gene expression fold change respect to the control leucocytes (unexposed). Statistically significant differences ($P<0.05$) between control and metal-exposed (*) were denoted.

Previous studies have already confirmed some immunotoxicological effects of metals in the European sea bass: *in vitro* and *in vivo* effects of Cd and Cu in HK macrophages (Bennani et al., 1996), *in vivo* effects of Cu in serum and blood leucocytes (Cotou et al., 2009), as well as *in vivo* effects of Cd or Hg in the number of HK macrophages (Giari et al., 2007, 2008). Therefore, we have exposed freshly isolated HKLs of European sea bass to concentrations higher and lower to the EC_{50} dosages found after 24 h of incubation but for shorter exposure time, 30 min or 2 h, which have already been used before and provoked immunotoxicological signs (Bennani et al., 1996; Vazzana et al., 2014; Witeska and Wakulska, 2007).

Firstly, we found that the cytotoxicity provoked by metals usually followed a dose-dependent curve and apoptosis was the main cell death mechanism induced, which agrees with the literature, including European sea bass leucocytes (Sarmento et al., 2004; Vazzana et al., 2014). We found that toxicity after 2 h exposure to metals was highest with Hg, followed by MeHg, Cd, As and Pb similarly to that found for gilthead seabream HKLs (unpublished data obtained in our group). Furthermore, generation of ROS induced by metals has been shown to be one of the predominant factors triggering cell death, mainly apoptosis (Rana, 2008). In this sense, ROS production has been demonstrated in fish cell lines exposed to metals such as Pb, Hg, As, Cu or Zn (Ariza et al., 1998; Fernández-Cruz et al., 2012; Sandrini et al., 2009; Selvaraj et al., 2013). Thus, potential interaction between oxidative stress and cell death was studied herein. Our data showed that Cd and Hg forms induced a significant ROS production and at the same time induced apoptosis after 30min and necrosis after 2 h exposure. This could probably be due to the fast and drastic cell death provoked by these metals, resulting permeable to PI after 2 h, and being late apoptotic cells more than necrotic cells. These results are in agreement with studies in rainbow trout cell lines exposed to Cd (Krumschnabel et al., 2010) or murine macrophages exposed to Hg (Kim and Sharma, 2004) that induces apoptosis and necroptosis at the same time. Furthermore, up-regulation in pro-apoptotic *bax* and *casp3* gene expression after HKLs exposure to Cd or Hg forms suggests that BAX protein and ROS provoke the translocation of cytochrome C from the mitochondria to the cytosol and activation of caspase-3, which is in concordance with the literature in rainbow trout or mammals (Cai et al., 2004; Kim and Sharma, 2003; López et al., 2003; Risso-De Faverney et al., 2004). Surprisingly, Pb triggered cell death by apoptosis in a dose-time dependent manner and

a significant up-regulation in *bax* and *casp3* gene expression without ROS production. This has been previously documented for Pb, which provoked apoptosis by opening the mitochondrial permeability transition pore (PTP) that didn't open due to oxidative stress (He et al., 2000). With respect to As, ROS production and apoptosis were both induced but at different concentrations, concomitant to a down-regulation of *bax* and *casp3* genes. In contrast, recent studies have demonstrated that As_2O_3 induced apoptosis and necrosis in a fish cell line (Selvaraj et al., 2013) through the depletion of intracellular glutathione (Li et al., 2006). In addition, we evaluated the expression of genes related to the ROS metabolism. ROS induction and up-regulation of *sod* and *cat* gene transcription was only concomitant in the case of European sea bass HKLs exposed to Hg forms. However, the increased ROS by Cd and As was not parallel to changes in *sod* and *cat* transcription. Moreover, Hg forms are the most potent ROS inducers in sea bass HKLs whilst As is in the gilthead seabream HKLs and SAF-1 cell line (unpublished data obtained in our group). In addition, the direct relation of ROS production and SOD and CAT, at either gene transcription or enzymatic activity, has not always found.

Other mechanisms have been shown to be important in the cellular protection against metals such as MTs (Bourdineaud et al., 2006) and HSP (Morimoto, 2011). Regarding anti-stress proteins, MTs are the first defense mechanism against the toxicity of metals, but when metal concentrations reaches high levels and produce protein denaturation, HSPs act to repair the damage (Giudice et al., 1999). Our results showed a significant increase of the *mta* gene expression after Cd and Pb exposure and surprisingly significant decrease after MeHg exposure in European sea bass HK leucocytes. This enhanced *mta*, after Cd and Pb exposure, is parallel to low or mid production of ROS and suggest the ability of MTA to partially scavenge ROS (Klaassen et al., 1999). In fact, MeHg exposure of sea bass HKLs produced a significant down-regulation of *mta* and *hsp70* protective mechanism, which was coincident with the highest ROS production. In the case of *hsp70* gene expression, no differences were found after Cd, Pb or As exposure in sea bass HKLs, which has been also documented for sea bass PBLs exposed to Cd (Vazzana et al., 2014). The predicted relationships among fish MT, HSPs and ROS production, and their role in toxicology, should focus further attention in.

Among the potential immunotoxicological effects on fish leucocytes, we investigated the impact of metals on *in vitro* European sea bass HKLs phagocytosis and respiratory burst activity. Thus, our results showed that the phagocytic ability, phagocytic capacity and the respiratory burst increased or decreased depending on the metal, dosage and exposure time. Though Cd, As and MeHg significantly decreased the phagocytic ability and capacity it is worthy to note that Pb and $HgCl_2$ produced an increment even with the knowledge that they suffered oxidative stress and cell death. Strikingly, our data show that all the metals also induced the respiratory burst activity. However, considering that different immunomodulatory effects of metals in mammals as well as fish depends on dose, mode, time and source of metal exposure, punctual contradictory results are not surprising. It has been reported that *in vitro* exposure of European sea bass head-kidney macrophages to $HgCl_2$ (Sarmento et al., 2004) and rainbow trout leucocytes, from blood or head-kidney, exposed to MeHg or $HgCl_2$ (Voccia et al., 1994) failed to affect the phagocytosis unless viability was reduced. By contrast, phagocytosis was decreased by *in vitro* exposure to Cd, Pb, As and Hg in murine macrophages (Christensen et al., 1993; Goering et al., 2000; Kasten-Jolly and Lawrance, 2014; Sengupta and Bishayi, 2002). Regarding the respiratory burst, our data are in sharp contrast to the literature in which the PMA-stimulated production of ROS was decreased in fish leucocytes exposed to Cr, Cu, Zn or Cd (Bennani et al., 1996; Sarmento et al., 2004; Steinhagen et al., 2004; Witeska and Wakulska, 2007) and mammalian macrophages (Irato et al., 2001; Palmieri et al., 2007). Interestingly, in a parallel study using gilthead seabream HKLs exposed to the same conditions than in this work, we found that Cd, Hg forms and As increased phagocytic ability and reduced phagocytic capacity and respiratory burst activities, except Pb that increased all the phagocyte parameters. However, there are not evidences demonstrating the mechanism by which the metals alter the phagocyte functions. In addition, the differences found between *in vitro* and *in vivo* immunotoxicological effects might be due to the differentiation state of the macrophages and the presence of other factors such as the serum levels of corticosteroids and catecholamines that do not operate *in vitro* (Walsh et al., 2002) a suggestion that is worthy of investigation.

Very little is known about the fish immunotoxicology of metals at gene level. Thus, we have evaluated some important genes involved in fish immunity after 2 h of exposure. Surprisingly, only small changes in the expression of immune-related genes were

provoked in the European sea bass HKLs exposed to metals. These changes included significant up-regulation of *phox40* (Pb) and *il1b* (Hg) and down-regulation of *mx* (Cd), *phox40* and *hepc* (MeHg) and *ighm* and *hepc* (Hg) mRNA levels. Our data suggest that B and T lymphocytes are not significantly altered, since the expression of their markers was not affected, except by Hg, but they have been shown to be more sensible to metals than phagocytes in common carp (Witeska and Wakulska, 2007). In fact, fish lymphocyte *in vitro* proliferation is commonly depressed by Zn, Mn, Hg forms or Cr (Ghanmi et al., 1989; Steinhagen et al., 2004; Voccia et al., 1994) and this should be followed by decreased expression of their cellular markers. Rainbow trout macrophages exposed to Cu up-regulated the gene expression of the pro-inflammatory cytokines *il1b* and tumor necrosis factor-α (*tnfα*) (Teles et al., 2011), suggesting some phagocyte activation. In the sea bass HKLs, only the exposure to $HgCl_2$ up-regulated the *il1b* transcription and this metal also induced the phagocytic ability suggesting a connection. Interestingly, As and Pb exposure induced the activity of the nuclear factor kappa B (NF-κB) in mammals (Chen et al., 2001; Woods et al., 2002) and this is responsible for the activation of cytokine production and many leucocyte functions. Therefore, this connection merits further evaluation in fish. However, this is not supported by the finding that the gene expression of the antibacterial *hepc* is down-regulated by both Hg forms, which also reduced the phagocytosis. Interestingly, *phox40*, involved in the regulation of the respiratory burst, gene expression was significantly induced towards an increment in the PMA-induced ROS production but the opposite did not occur. Thus, our data suggest, at least, a positive relation between these two parameters. Regarding this, though it is generally observed an inhibition of the respiratory burst in fish leucocytes after metal exposure a unique study has found increased *phox* transcription after Cu exposure (Teles et al., 2011). Further characterization of the relation among NADH oxidase components and the respiratory burst is needed in fish leucocytes, at least under toxicological condition.

General discussion

From the perspective of ecotoxicants, fish are especially important (Bols et al., 2001). With more than 30,000 different species occupying all aquatic niches, fish are the first and most diverse group of vertebrates. Thus, understanding the actions of ecotoxicants on fish helps in the evaluation of the aquatic environment health. On the other hand, the use of alternative methods has increased in recent years to comply with the strategy of 3Rs (reduction, refinement and replacement) (Sonali et al., 2015). Thus, cell lines and primary cell cultures derived from fish represent technically easy to handle and reproducible test systems that provide very good correlation with acute lethality tests *in vivo* (Segner, 2004). Most of the information focusing on fish toxicology comes from freshwater species and less is known about marine fish species and their cell lines. Indeed, very few established cell lines or primary cell cultures have been developed from marine cultured fishes such as gilthead seabream (*S. aurata)* or European sea bass (*D. labrax*), the most important Mediterranean cultured fish species.

1. Cytotoxicity

1.1. Fish cell lines

We have evaluated the cytotoxicity of metals on two fish cell lines, one generated in this work from European sea bass brain (DLB-1) and another commercially available from gilthead seabream fins (SAF-1). Firstly, the DLB-1 cell line has been obtained form brain explants and after infection with fish retrovirus. Actually, the cell line is cultured for more than two years and has around 100 passages. Cell morphology is epithelioid, with some star-like cells that are more abundant until the confluence is achieved. Cells show a rapid growth and the expression of cellular markers indicated us that they are of glial origin though further studies are now in progress to confirm this. In the literature, some other brain-derived cell lines have been described with differences or controversies in their growth, origin and morphology (Ahmed et al., 2009; Bloch et al., 2015; Chen et al., 2010; Chi et al., 2005; Fu et al., 2015; Hasoon et al., 2011; Ku et al., 2009; Lai et al., 2001, Lai et al., 2003; Servili et al., 2009; Wang et al., 2010; Wen et al., 2008a, b; Zheng et al., 2015). Strikingly, a long-term culture of European sea bass brain was described earlier but no information has been added since then (Servili et al.,

2009). These cells resemble to be neural stem cells and showed slow growth rates. Unfortunately, very few data exist regarding the toxicological application of such brain-derived cell lines.

For evaluating the cytotoxicity of metals *in vitro* on DLB-1 and SAF-1 fish cell lines, we used some colorimetric methods such as NR, MTT, LDH release or CV. These methods allowed us to evaluate lysosomal damage, mitochondrial impairment, membrane integrity and cell attachment/detachment, respectively. Thus, the viability measured after 24 h exposure to metals showed a dose-dependent reduction of the number of viable cells in both fish cell lines, whatever detection method used. These results are in agreement with studies performed with other fish cell lines, in which the viability decreases after metal exposure (Babich and Borefreund, 1991; Bayoumi et al., 1999; Segner, 1998; Minghetti et al., 2011). These results are not surprising if we are aware of the toxic nature of them.

If we compare the acute EC_{50} values (the lethal concentration required to kill 50% of the population) clearly showed that DLB-1 cell line is more susceptible to metals than SAF-1, whatever the detection method used (Table II).

Table II. Values of EC_{50} (mM) after exposure of SAF-1 and DLB-1 cells to metals for 24 h. NR, neutral red, MTT, yellow soluble tetrazolium salt; CV, crystal violet; LDH, lactate dehydrogenase.

Metals	SAF-1 cell line				DLB-1 cell line	
	NR	MTT	CV	LDH	NR	MTT
Cd	0.1	0.1	0.160	0.703	0.004	0.015
MeHg	0.015	0.018	0.021	0.018	0.013	0.020
Pb	3.2	3.4	4.5	4.2	1.6	1.5
As	0.082	0.03	0.367	0.226	0.03	0.018

Differences in cell lines susceptibility could be due to the source of the cell lines (fish species or tissue). Thereby, SAF-1 cells are fibroblastic cells derived from fin explants

of seabream (Béjar et al., 1997), while DLB-1 are glial cells from sea bass brain. Nervous system cells have a variety of unique structural characteristics that likely make them more sensitive to toxics than other cells. They require a high aerobic metabolic rate to provide them high levels of oxygen, glucose, proteins and lipids to maintain ion gradients to support neuronal transmission. Although glia cell does not directly participate in synaptic interactions and electrical signalling, their supportive functions help to define synaptic contacts and maintain the signalling abilities of neurons (Perea et al., 2014). Indeed, some authors have described impaired human or rat glial cells after Pb (Tiffany-Castiglioni, 1993), Al (Lukiw and McLachlan, 1995), Hg (Zhaobao, 2011; Monnet-Tschudi et al., 1996), Cd (Choi et al., 2002) or As exposure (Cheng et al., 2004; Wang et al., 2011).

The cytotoxic assays used revealed different profiles between the detection method, NR, MTT, CV or LDH, but in both cell lines NR was the most sensitive technique used, following MTT, CV and LDH in the case of SAF-1 cells. Indeed, LDH or CV was only suitable after exposure to high metal concentrations or long time in SAF-1 cell lines. This is supported by several authors (Minghetti et al., 2011; Segner, 1998), who found that the EC_{50} values are usually higher for LDH leakage assay and protein assays (like the CV method) than for NR and MTT assays. This demonstrates that organelle impairment occurs before any permanent cell membrane damage and cell detachment alteration. For Cd, MeHg and Pb, the NR and MTT curves were almost identical in the SAF-1 cell line, and gave very similar EC_{50} values, suggesting that both lysosomes and mitochondrial organelles are impaired at similar metal concentrations (Cornelis et al., 1992; Maracine and Segner, 1998; Vian et al., 1995); however, except Pb, Cd, MeHg and As curves were slightly different in DLB-1 cells, suggesting that lysosomes are altered earlier than mitochondria organelles. Likely, the origin of the cells and their characteristics may also determine the mechanisms of metal toxicity. Surprisingly, in both fish cell lines, mitochondria were the most affected organelle after As exposure. This can be partly explained by the fact than inhibition of mitochondrial respiration is regarded as a primary mechanism by which As manifests cell injury (Rana, 2008).

In addition, the EC_{50} values indicated that MeHg was the most toxic metal for SAF-1 as shown in other fish cell lines studies (Maracine and Segner, 1998) whilst Cd was the most toxic for DLB-1 cells. Although MeHg is well known to be a potent neurotoxic, the role of other metals such as Cd in the nervous system is still unclear. Cd is thought

to act disrupting neurotransmitter uptake (Beauvais et al., 2001). Given that Cd induced a high ROS level in DLB-1 as we will discuss later, this could partly explain the high cytotoxicity of Cd in DLB-1 cells. On the other hand, the cytotoxic capacity of Pb was the lowest compared to other metals for either cell lines, which is confirmed by other studies with a similar pattern in RTG-2 or BF-2 cell lines (Bayoumi et al., 1999; Maracine and Segner, 1998).

1.2. Primary cell cultures

For evaluating the *in vitro* cytotoxicity using primary cell cultures, we choose three primary types of cell cultures: HKLs, PBLs and erythrocytes from marine gilthead seabream and European sea bass specimens. Thereby, primary cell cultures were exposed *in vitro* for 24 h to Cd, MeHg, Pb and As and the cytotoxicity was evaluated. For this purpose, we used fluorescent dye PI, which with the loss of the membrane integrity, it is taken-up and complexes with nuclear DNA, which emits red fluorescence (Ormerod, 1990). This method is preferred since we use heterogeneous populations (lymphocytes, monocyte-macrophages and granulocytes) containing different amounts of mitochondria and lysosomes, which could affect to the MTT and NR assays. For erythrocytes, apart from this, we also used another technique based on the release of HbO_2 after hemolysis of erythrocytes (Martínez-López et al., 2005). The cytotoxicity assays in erythrocytes revealed similar profiles with the two techniques, thus, we represent only the PI values.

As we have noted with fish cell lines, the PI cytotoxic method showed a dose-dependent curve after 24 h exposure to the metals in HKLs, PBLs and erythrocytes from gilthead seabream and European sea bass. First of all, if we compare the acute EC_{50} values, we observed that MeHg was the most toxic metal, followed by Cd, As and Pb after 24 h of exposure. These results are in agreement with the cytotoxicity values and pattern obtained on seabream and sea bass HKLs exposed to metals for 2 h (Table I2).

Furthermore, we found that the seabream cells are more sensitive than those from sea bass when they are exposed to metals. The toxicity order within the same fish species is erythrocytes> HKLs> PBLs. A potential explanation of this could be due to the fact that erythrocyte membrane differs from other membranes in its very high anion permeability and this fact has been related with the more avid uptake of metals by erythrocytes

(Foulkes, 2000). We suggest that wide variations are found in the permeability properties of cells from different tissues. Even the transport of metals may vary significantly from one cell type to another.

Table 12. Values of EC_{50} (mM) of fish leucocytes and erythrocytes from seabream and sea bass after exposure to metals for 24 h. HKLs, head-kidney leucocytes; PBLs, peripheral blood leucocytes.

Metal						
	HKLs	**PBLs**	**Erythrocytes**	**HKLs**	**PBLs**	**Erythrocytes**
Cd	0.110	0.171	0.022	1.5	2.6	0.080
MeHg	0.005	0.012	0.021	0.3	0.464	0.028
Pb	2.8	2.9	0.523	3.4	4.2	1.1
As	0.218	0.491	0.134	2.7	3.8	0.190

The great differences among cell sources could be due to the very different cell types but also to the heterogeneity and abundance of mature and immature stages of the cells. Thus, HKLs is the most diverse and heterogeneous culture used and composed of leucocytes at all developmental stages (Press et al., 1991). However, PBLs is the most concrete population and formed by mature cells. Finally, our results showed that seabream cells were more sensitive to metals than sea bass cells, in sharp contrast to what happened with their respective cell lines.

In conclusion, the results show that metal exposure provokes a dose-dependent reduction in the viability of primary cell cultures, as we observed in the fish cell lines. Regarding fish cell lines, MeHg produced the highest toxicity in SAF-1 cells while Cd was the most toxic metal for DLB-1 cells, as well as Pb was the lowest toxic in both cell lines. In addition, NR was the most sensitive colorimetric method, followed by MTT, CV and LDH. Upon primary cell cultures, also MeHg produced the highest toxicity in HKLs, PBLs and erythrocytes from seabream and sea bass. Interestingly, leucocytes

and erythrocytes from European sea bass are more resistant to metal exposure than those from gilthead seabream. Similarly, erythrocytes, followed by HKLs, are always more sensitive than those isolated from blood from the same fish species.

2. Cellular protection and oxidative stress

Oxidative stress is an unavoidable aspect of aerobic life and is the result of an imbalance between the production of ROS and antioxidant defences in living organisms (Nishida, 2011). Furthermore, metals are important inducers of oxidative stress in fish, promoting the formation of ROS. Fortunately, cells have developed mechanisms of antioxidant defences to protect from them. In the present work, ROS production and expression of selected genes involved in cellular protection and antioxidant defences was also evaluated in SAF-1 and DLB-1 fish cell lines and HKLs, PBLs and erythrocyte primary cultures from seabream and sea bass after metal exposure. The results obtained with the different cell types are summarized in the Table 13, 14.

Table 13. ROS production in fish cell lines and primary cell cultures derived from gilthead seabream and European sea bass after metal exposure. The arrows indicate a significant increase (↑), decrease (↓) or no differences (↔) of the parameter studied compared to control. Not determined parameters (−).

Fish cells			ROS production			
			Cd	Hg	Pb	As
SAF-1			↑	↑↑	↑	↑↑↑
DLB-1			↑↑↑	↔	↑↑↑	↔
HKLs		30´-2h	↑	↑↑	↔	↑↑↑
		24h	−	−	−	−
		30´-2h	↑	↑	↔	↔
		24h	−	−	−	−

In all the fish cells which measured cellular oxidative stress after metal exposure, we observed a significant increase in the cellular ROS levels, as indicated by DHR 123 fluorescence. These results are confirmed in fish cell lines and fish primary cell cultures

exposed to Pb, Hg, As, Cu or Zn (Ariza et al., 1998; Fernandez et al., 2013; Kim and Sharma, 2004; Romero et al., 2003; Sandrini et al., 2009; Selvaraj et al., 2013). However, depending on the cell type and origin, metal or time-exposure, the cell stress response is different.

It is widely accepted that these ROS are the main inducers of the cytotoxicity (Benedetti et al., 2007; Loro et al., 2012; Monteiro et al., 2010; Wang et al., 1996). However, our data are not fully supporting this hypothesis. As alluded to earlier, in the case of SAF-1 cells, MeHg and Pb produced the highest and lowest cytotoxicity which does not agree with the overproduction or lower level of ROS after As or Cd and Pb exposure, respectively. Nevertheless, Cd is the most cytotoxic metal and overproduces ROS in DLB-1 cell line but Pb, which induces high levels of ROS production, is the less cytotoxic metal. Indeed, it is known that Pb is a potent neurotoxic that induce oxidative stress (De Gennaro, 2002).

Regarding HKLs exposure after a short time, we observed that MeHg and Pb produced the maximum and minimum cytotoxicity which agrees with overproduction and lower ROS levels by MeHg and Pb, respectively, in seabream and sea bass leucocytes. Furthermore, As induced an overproduction of ROS in seabream leucocytes as well as we observed in SAF-1 cells. These results suggest that ROS radicals are not the most important inducers of the cytotoxicity mediated by metals. We may conclude that all metals, especially As, provoke the overproduction of ROS in fish cells which agrees with studies in mammalian (Wang et al., 1996) and fish (Selvaraj et al., 2013; Wang et al., 2004). Moreover, it has been shown that the oxidation of As under physiological conditions results in H_2O_2 formation (Valko et al., 2005), what could partly explain the very high ROS levels in our assays. Interestingly, seabream leucocytes exposed to metals overproduced more ROS than sea bass leucocytes and this could also explain why sea bass leucocytes are more resistant to metals.

Likely, the modulation of mRNA transcription of genes involved in oxidative stress also affects the stress response. Thus, the generation of hydrogen peroxide is mainly produced at mitochondrial level by SOD while its elimination may involve CAT, GT and PRXs (Di Giulio and Meyer, 2008; Lushchak, 2011). In general, our results show that ROS production in fish cells exposed to metals is concomitant with the increased *sod* gene transcription and the scavengers of *cat*, *gr*, *prx1* and *prx2* (Table I4). However,

some contradictory results are observed depending on the metal and cell population. For example, Cd, MeHg and As exposure down-regulated *sod*, *cat* and *gr* transcription in seabream HKLs, while only *sod* transcription was down-regulated in sea bass HKLs after 24 h exposure. Surprisingly, As induced the highest ROS production and the lowest *sod, cat, gr, prx1 and prx2* transcription in SAF-1 cells supporting the idea that part of the ROS come from the direct As oxidation above mentioned. After short time exposure, *sod* and *cat* transcription was induced by metals in seabream HKLS while As exposure down-regulated *cat* gene expression. Furthermore, Cd, MeHg and As reduced the *cat* gene expression in seabream PBLs and erythrocytes. Thus, SOD, CAT and glutathione system have shown different patterns and some controversy in fish exposed to metals, but still considered good biomarkers of the oxidative stress produced by metals (Altikat et al., 2014; Bhattacharya and Bhattacharya, 2007; Di Giulio and Meyer, 2008). Furthermore, we demonstrated the role of the PRXs as biomarker, but no studies have evaluated it in fish exposed to metals.

Table 14. Gene expression in fish cell lines and primary cell cultures derived from gilthead seabream and European sea bass after metal exposure. The arrows indicate a significant increase (↑), decrease (↓) or no differences (↔) of the parameter studied compared to control. Not determined parameters (−).

Fish cells			Gene expression																									
			mta				*hsp70*				*prx1*				*prx2*				*sod*				*cat*				*gr*			
			Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As
SAF-1			↑	↑	↑	↓	↔	↑	↓	↓	↔	↑	↑	↔	↔	↑	↑	↓	↑	↑	↑	↓	↑	↑	↑	ND	ND	↑	↑	ND
DLB-1			↔	↑	↔	↑	–	–	–	–	–	–	–	–	–	–	–	–	↔	↔	↔	↔	↔	↓	↓	↓	–	–	–	–
HKLs		30´-2h	↑	↓	↔	↓	–	–	–	–	–	–	–	–	–	–	–	–	↔	↑	↔	↔	↑	↑	↔	↑	–	–	–	–
		24h	↔	↓	↔	↓	↔	↓	↔	↓	–	–	–	–	–	–	–	–	↓	↓	↔	↓	↓	↓	↔	↓	↓	↓	↔	↔
		30´-2h	↑	↓	↑	↔	↔	↓	↔	↔	–	–	–	–	–	–	–	–	↔	↑	↑	↔	↔	↑	↑	↓	–	–	–	–
		24h	↑	↔	↔	↑	↔	↔	↔	↑	–	–	–	–	–	–	–	–	↔	↓	↔	↔	↔	↑	↔	↑	↔	↔	↑	↑
PBLs			↔	↓	↔	↔	↔	↑	↑	↔	–	–	–	–	–	–	–	–	↑	↑	↔	↔	↓	↓	↔	↔	↔	↔	↔	↔
			↑	↑	↔	↑	↑	↑	↔	↑	–	–	–	–	–	–	–	–	↔	↑	↔	↑	↑	↑	↔	↑	↔	↔	↔	↑
Erythrocytes			↑	↑	↔	↔	↑	↑	↑	↑	↑	↑	↑	↔	–	–	–	–	↔	↑	↑	↑	↓	↓	↔	↓	↓	↓	↔	↓
			↔	↑	↔	↔	↔	↔	↓	↑	↑	↔	↔	↑	–	–	–	–	↑	↑	↑	↑	↔	↔	↔	↑	↑	↑	↔	↑

Similarly, our results showed a regulation of *mta* and *hsp70* transcription after metal exposure in all the cell populations in which they were evaluated. It is important to

consider that these are most important proteins protecting cells against metal toxicity, especially Cd, Hg, Cu and Zn. In addition, MT has been shown to directly react with hydroxyl radicals in cell-free *in vitro* systems (Thornalley and Vasak, 1985) and may, consequently, act as a donor of reduced thiolate groups, thereby fulfilling a role in protecting the cell from free radical mediated injury and reducing oxidative stress (Lazo et al., 1995; Ruttkay-Nedecky et al., 2013). In general, *mta* was up-regulated by Cd, MeHg or to some extent by Pb and As in all cell types, and specially down-regulated mainly by MeHg or As in all primary cell cultures and SAF-1 cell line. Indeed, Cd and Hg bind easily to MTs (Bebianno et al., 2007; Roméo et al., 2000; Shaw et al., 1991), but an excess of metals could provoke a MT dysfunction leading to an increase of ROS levels as we have observed in some cells after metal exposure. Upon heat-shock proteins, *hsp70* gene expression followed a quite parallel pattern demonstrating cell injury, whilst As or MeHg produced a down-regulation of *hsp70 in* SAF-1 cells or seabream and sea bass HKLs exposed for short time. Surprisingly, all blood cells showed a strong up-regulation of *mta* and *hsp70* transcription. An explanation of this could be the fact that since metals are absorbed mainly by gills in fish, they are distributed by blood to the different tissues bound to cysteine residues of MTs. Thus, not only erythrocytes but also PBLs could contribute to increase the tolerance of fish to metals, but also decrease metal excretion rates due to sequestration.

To conclude, the present results showed a moderate production of ROS after metal exposure in a dose- and time-dependent manner in all cell populations. However, depending on the metal, the induction of ROS levels was highest or lowest. Similarly, the regulation of genes involved in oxidative stress and cell protection suggested the activation of mechanisms to equilibrate the imbalance of oxygen radicals after metal exposure. However, an excess of metal levels in cells could trigger the inactivation of antioxidant defences. This could be happening with MeHg and As, which are the most harmful metals in all the cell populations due to the induced high levels of ROS and impaired most of the enzyme defence mechanisms.

3. Cell death

It is well-known that a consequence of imbalanced oxidative stress caused by metals is cell death by apoptosis (Rana, 2008). However, few papers have dealt with this aspect in fish cell lines or primary cell cultures. Thus, in the present work, we have studied the cell death mechanisms after metal exposure in fish cells. To carry out this, morphological changes, abundance of cells in apoptosis and/or necrosis and the expression of apoptotic regulatory genes were evaluated. Thus, the results are showed in the Table 15.

Table 15. Cell death via apoptosis (A) or necrosis (N) and expression of genes related to apoptosis after metal exposure in fish cell lines and primary cell cultures derived from gilthead seabream and European sea bass. The arrows indicate a significant increase (↑), decrease (↓) or no differences (↔) of the parameter studied compared to control. Not determined parameters (–).

Fish cells			Cell death				Gene expression											
			Cd	MeHg	Pb	As	*bcl2*				*bax*				*casp3*			
							Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As
SAF-1			A+N	A+N	A+N	A+N	↓	↓	↓	↓	↑	↑	↑	↔	–	–	–	–
DLB-1			A	A	A	A	↓	↓	↓	↓	–	–	–	–	–	–	–	–
HKLs		30´-2h	A	A+N	A	A	↔	↑	↓	↔	↔	↓	↑	↔	↔	↔	↔	↔
		24h	–	–	–	–	–	–	–	–	↑	↑	↔	↑	↑	↑	↔	↑
		30´-2h	A+N	A+N	A	A	–	–	–	–	↔	↑	↑	↓	↔	↑	↑	↓
		24h	–	–	–	–	–	–	–	–	↑	↔	↔	↑	↔	↔	↔	↑
PBLs			–	–	–	–	–	–	–	–	↔	↔	↑	↔	↑	↑	↔	↑
			–	–	–	–	–	–	–	–	↔	↑	↔	↑	↔	↑	↔	↑
Erythrocytes			–	–	–	–	–	–	–	–	↔	↔	↑	↑	–	–	–	–
			–	–	–	–	–	–	–	–	↔	↔	↔	↑	–	–	–	–

Regardless of the technique used and the origin of the cells, our data demonstrated that apoptosis is the main cell death mechanism induced by all the metals tested as previously documented in fish leucocytes (Vazzana et al., 2014) and cell lines (Selvaraj et al., 2013), as well as in mammals (Rana, 2008). However, characteristics of necrosis

cannot be completely excluded. Indeed, SAF-1 cells treated showed abundant blebs in the membrane resembling those produced in apoptosis cell-death, whilst at the same time other cells showed holes in the membrane or even lost their membrane, indicating that they were in very late apoptosis or necrosis. Similarly, DLB-1 cells exposed to EC_{50} of metals also died by apoptosis as demonstrated by the increase of sub-G0/G1 in the cell cycle and Annexin staining. Furthermore, seabream and sea bass HKLs exposed to Cd or MeHg showed a very fast cell apoptosis after 30 min of incubation, but no after 2 h, followed by an increment of necrotic cells after 2 h. Likely, due to the high permeability, metals may provoke a fast and drastic cell death, and the necrotic cells represent late apoptotic cells more than cells directly killed by a necrosis process. In a similar way, some reports have also documented the induction of apoptosis and necroptosis in fish and mammals (Kim and Sharma, 2004; Krumschnabel et al., 2010; Rana, 2008; Selvaraj et al., 2013). Furthermore, lipid peroxidation is perhaps the most studied consequence of ROS (Devyatkin et al., 2006), which can produce alterations in cell membrane, and finally, necrosis and this could also be happening. Moreover, the expression of apoptosis regulatory genes confirms these findings. Thus, pro-apoptotic *bax* gene was up- regulated, except by As (in SAF-1 cells, seabream and sea bass HKLs) and by MeHg (in seabream HKLs) and the anti-apoptotic *bcl2* down- regulated (except after MeHg exposure to seabream HKLs) by metals confirming this cell death pathway. In addition, the up-regulation of the *casp3* suggested that BAX protein and ROS provoke the translocation of cytochrome C from the mitochondria to the cytosol, activation of caspase 3, and finally apoptosis cell death, which is in concordance with the literature in rainbow trout or mammalian cells (Risso-De Faverney et al., 2004). Similarly, a significant up-regulation of *bax* transcription after exposure to Pb and As in erythrocytes from seabream and sea bass species suggested that these metals induced apoptosis cell death; however, Cd and MeHg did no alter its mRNA levels. Thus, these metals could not trigger apoptosis, or alter other genes involved in the regulation of apoptosis in erythrocytes from both species.

Thus, to conclude, our data demonstrated that metals induce cell death mainly by apoptosis. These results are supported by the up-regulation of *bax* and *casp3* and down-regulation of *bcl2* gene transcription.

4. Immunotoxicology

Few studies have evaluated the immunotoxicological effects of the toxicants *in vitro* because there are very few available immune-related fish cell lines and the use of freshly isolated leucocytes has attracted little interest among researchers. Strikingly, it has been quite well correlated the cytotoxicity assays in fish cell lines with acute lethality test in fish (Segner, 2004), and it would be interesting to evaluate whether this relation between the effects of toxicants on isolated leucocytes responses and immunotoxicology *in vivo* could be established in fish as well. Interestingly, European sea bass leucocytes reduced the phagocyte functions [respiratory burst, phagocytosis or the benefits of macrophage-activating factors (MAF)] by *in vitro* exposure to Hg or Cu (Bennani et al., 1996; Sarmento et al., 2004), whilst Cd exposure led to their increment (Bennani et al., 1996). However, no studies have been conducted with gilthead seabream leucocytes *in vitro*. Similarly, very few papers have dealt with the toxicological mechanisms caused by metals at gene level. Thus, in the present work, we evaluated the phagocytosis and the respiratory burst activity (two immune responses) of head-kidney leucocytes (HKLs) derived from gilthead seabream and European sea bass after 30 min or 2 h of metal exposure. Furthermore, we performed the analysis of immune relevant genes in gilthead seabream and European sea bass HKLs and PBLs after 24 h. The results are summarized in the Table I6 and I7. Thus, our results show that the phagocytic ability, phagocytic capacity and the respiratory burst increased or decreased depending on the metal, dosage and exposure time in gilthead seabream and European sea bass leucocytes (Table I6).

While MeHg increased the phagocytic ability in seabream leucocytes, in sea bass leucocytes the ability was impaired by Cd, MeHg and As. Surprisingly, Pb increased the phagocytic ability in both species in a dose-dependent manner. However, all metals reduced the phagocytic capacity except Pb, which enhanced it at low dosages in seabream leucocytes. It is worthy to note that Pb and Hg produced an increment even with the knowledge that they induced oxidative stress and cell death as we described above. These contradictory results are not surprising if we observe the literature. For example, Cd increased the phagocytosis in gilthead seabream specimens exposed *in vivo* to waterborne As, Cd (Guardiola et al., 2013a, b) or MeHg (Guardiola et al., 2016).

Table 16. Phagocytosis activity or respiratory burst activity in seabream and sea bass HKLs and PBLs after metal exposure. The arrows indicate a significant increase (↑), decrease (↓) or no differences (↔) of the parameter studied compared to control. No determined parameters (–).

Fish cells			Phagocytosis								Respiratory burst			
			Ability				Capacity							
			Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As
HKLs		30´-2h	↔	↑	↑	↔	↔	↓	↑	↓	↓	↓	↑	↓
		24 h	–	–	–	–	–	–	–	–	–	–	–	–
		30´-2h	↓	↓	↑	↓	↓	↓	↓	↓	↔	↓	↑	↓
		24 h	–	–	–	–	–	–	–	–	–	–	–	–

Thus, the presence of factors such as the serum levels of corticosteroids and catecholamines that do not operate *in vitro* could explain the differences observed between *in vitro* and *in vivo* (Randall and Perry, 1992).

Phagocytic activity was unaffected in European sea bass head-kidney macrophages exposed to $HgCl_2$ (Sarmento et al., 2004) and in rainbow trout leucocytes, from blood or head-kidney, exposed to MeHg or $HgCl_2$, except at those concentrations provoking leucocyte death (Voccia et al., 1994). By contrast, phagocytosis activity was decreased by *in vitro* exposure to Cd, Pb, As and Hg in murine macrophages (Christensen et al., 1993; Goering et al., 2000; Kasten-Jolly and Lawrence, 2014; Sengupta and Bishayi, 2002). However, the mechanisms by which metals alter the phagocyte functions are still poorly understood. A potential explanation could be that seabream phagocytic cells consist mainly of acidophilic granulocytes and they could be modulated in a different way than macrophages. In addition, the increased phagocytic ability of leucocytes exposed to Pb and Hg could be due to the increase of apoptotic bodies in the samples, which are ingested by phagocytes (De Almeida and Linden, 2005). Likely, some seabream and sea bass phagocytes are killed by metals as we expected whilst others are greatly activated to exert their biological function but these needs to be verified. However, though more phagocytes are activated, they are able to ingest fewer particles,

except those exposed to Pb. This could be attributed to alteration in the cell-surface receptors mediating the phagocytosis process but other kind of studies should confirm if this is happening.

On the other hand, ROS are produced by macrophages as cytotoxic agents against pathogens during the respiratory burst, via stimulation of the NADPH oxidase system. In the respiratory burst activity, the production of ROS by granulocytes and macrophages was measured after PMA-activation of the NADH oxidase, radicals that are employed in the phagocytic activity of these cells to kill the pathogens (Babior, 2000). Our results show that except Pb, all the rest of metals impaired PMA-induced ROS production in seabream and sea bass leucocytes. *In vivo* studies, waterborne exposure to As, Cd or MeHg produced an increase, reduction or no alteration, respectively, in the seabream HKLs respiratory burst (Guardiola et al., 2013a, b, 2016). Our data agree with most of those obtained *in vitro* in which the PMA-stimulated production of ROS was decreased in fish leucocytes exposed to Cr, Cu, Zn or Cd (Bennani et al., 1996; Sarmento et al., 2004; Steinhagen et al., 2004; Witeska and Wakulska, 2007), or in mammalian macrophages exposed to Cd or As (Irato et al., 2001; Palmieri et al., 2007). In addition, in our study, two markers of the NADH oxidase, *phox22* and *phox40*, were evaluated. Divergent results were observed depending on the metal. Thus, we found up-regulation in seabream HKLs transcription of *phox22* and/or *phox40* by Cd and down-regulation by mercury while Pb induced an increase of *phox40* gene expression and mercury down-regulation in sea bass leucocytes (Table 17). Despite the inhibitory effects of metals in the respiratory burst in fish leucocytes, an increase of *phox* transcription after Cu exposure in rainbow trout macrophages was also evidenced (Teles et al., 2011). The activation of NADH system triggers the ROS production, named respiratory burst, thus, a correlation between these two parameters need further analysis.

In order to cast some light on the immune mechanisms leading to the immunotoxicology of metals we evaluated the expression of some immune-related genes (Table 17). Our results show that the mRNA transcription was regulated by the metal exposure and the origin of leucocytes. Surprisingly, only small changes in the expression of immune-related genes were observed in the European sea bass HKLs exposed to metals compared to seabream.

Table 17. Expression of immune-related genes in gilthead seabream and European sea bass HKLs and PBLs after metal exposure. The arrows indicate a significant increase (↑), decrease (↓) or no differences (↔) of the parameter studied compared to control. Not determined parameters (–).

Fish cells			Gene expression																											
			ighm				*tcrb*				*mx*				*bd*				*hepc*				*il1b*				*phox22*				*phox40*			
			Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As	Cd	Hg	Pb	As
HKLs		30´-2h	↑	↔	↔	↔	↔	↓	↔	↔	↔	↔	↔	↔	↔	↑	↔	↔	–	–	–	–	↔	↓	↔	↑	↑	↓		↔	↑	↓	↔	↔
		24h	↓	↓	↔	↓	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	↓	↓	↓	↓	–	–	–	–	–	–	–	–
		30´-2h	↔	↔	↔	↔	↔	↔	↔	↔	↓	↔	↔	↔	–	–	–	–	↔	↓	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓	↑	↔
		24h	↔	↔	↔	↔	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	↔	↔	↔	↔	–	–	–	–	–	–	–	–
PBLs			↓	↓	↔	↓	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	↓	↓	↓	↓	–	–	–	–	–	–	–	–
			↔	↔	↓	↔	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	↔	↔	↓	↓	–	–	–	–	–	–	–	–

Our data showed increased B lymphocyte marker (*ighm*) by Cd suggesting a stimulation of cell proliferation and a decrease in the T and B markers (*ighm* and *tcrb*, respectively) by MeHg exposure in sea bass leucocytes, which agree with the reduced lymphoproliferation of fish leucocytes after mercury exposure (MacDougal et al., 1996; Voccia et al., 1994) or after Zn, Mn, or Cr exposure (Ghanmi et al., 1989; Steinhagen et al., 2004). Similarly, seabream HKLs and PBLs showed a down-regulation of *ighm* transcription after 24 h exposure to Cd, MeHg or As. However, we failed to observe any change in the *ighm* and *tcrb* mRNA levels of sea bass leucocytes. We also evaluated the transcription of antiviral and antibacterial effector molecules, Mx, beta-defensin and hepcidin, respectively. While *mx* and *bd* genes were up-regulated by MeHg in seabream leucocytes suggesting that phagocytes are not greatly impaired in their immune functions, *mx* and *hepc* gene expression was reduced by Cd and MeHg exposure respectively, in sea bass leucocytes. Moreover, the pro-inflammatory cytokine *il1b* gene expression was up-regulated by As and down-regulated by Cd and mercury. This cytokine, among others, was up-regulated by Cu exposure in rainbow trout head-kidney macrophages (Teles et al., 2011). Thus, depending on the metal, the seabream leucocytes were, or not, under an inflammatory response. However, as seen earlier, *il1b* gene expression was not altered in sea bass leucocytes. Furthermore, after 24 h exposure to metals, *il1b* was down regulated in seabream and sea bass HKLs and PBLs. As we

discussed before, after 24 h of metal exposure, many cells are in apoptotic cell death process, thus many immune activities might be impaired.

To conclude, the present results show that HKLs from gilthead seabream and European sea bass exposed to Cd, Hg or As for 30 min or 2 h have impaired phagocytosis and respiratory burst immune parameters. Furthermore, immune relevant genes also were altered after metal exposure. These results are not surprising if we take into account that metals can modulate the immune functions of leucocytes, as we discussed before. Surprisingly, Pb was able to induce phagocytic ability, capacity and respiratory burst in both species, however, no alterations in gene expression were observed. Thereby, the scarce available data using freshly isolated fish leucocytes *in vitro* suggests that they could be useful to extrapolate and understand the immunotoxicological impact of pollutants *in vivo* and should be further explored. Additional studies are still required to understand the mechanisms involved for the immunotoxicological effects of different metals and their implications in fish biology and potential impacts in aquaculture.

Conclusions

The main conclusions of the present Thesis are:

1. A new European sea bass brain cell line, called DLB-1, which shows glial origin, rapid cell division and low transfection rate has been generated. More efforts should be done to develop fish cell lines for Cellular and Molecular studies.

2. Exposure to metals Cd, MeHg, Pb and As resulted in cytotoxicity in a dose-dependent manner in all the fish cells studied. MeHg produced the highest cytotoxicity, except for DLB-1 cells, while Pb was the lowest toxic metal.

3. Among the primary cell cultures, erythrocytes show the highest cytotoxicity after metal exposure followed by head-kidney and blood leucocytes.

4. Regarding fish species, European sea bass exhibit more resistance to metal exposure than gilthead seabream.

5. Cellular protection and antioxidant defences are induced in fish cells after metal exposure but they are unable to compensate the ROS overproduction leading to a marked oxidative stress, which in turns induces the cellular death, mainly by apoptosis.

6. Immune response is impaired in European sea bass and gilthead seabream head-kidney leucocytes after metal exposure at both gene and functional levels.

7. Both fish cell lines and primary cell cultures represent valuable tools to evaluate the mechanisms of metal toxicity.

8. Gilthead seabream and European sea bass erythrocytes should be further used as *in vitro* models in Toxicological studies.

Resumen en castellano

1. Introducción

En las últimas décadas, uno de los sectores empresariales con gran auge y proyección de futuro es la acuicultura, la cual tiene por objeto proveer alimento para el consumo humano a la vez que intentar suplir el descenso de las capturas de individuos silvestres (FAO, 2012). En toda la región Mediterránea, y concretamente en la región de Murcia, este sector se centra principalmente en dos especies marinas, la dorada (*S. aurata*), con una producción de 3.730 toneladas (22 % del total de la producción en España), y la lubina (*D. labrax*), encabezada por la Región de Murcia nivel nacional, con una producción de 4.987 toneladas (el 34% del total de producción en España) (FAO, 2012).

Son numerosos los aspectos físicos, químicos y biológicos a tener en cuenta en el cultivo intensivo de estos animales, además de las condiciones higiénico-sanitarias que éstos deben tener para satisfacer el consumo humano. Numerosos estudios han confirmado que diversas enfermedades y factores de estrés derivados del manejo y cultivo intensivo de peces provocan graves pérdidas económicas en el sector acuícola. Sin embargo, es menos conocido el efecto adverso que la contaminación acuática puede acarrear a la producción. Pero, además, este hecho es fundamental ya que los peces actúan como bioacumuladores de muchos contaminantes ambientales que luego pueden pasar al hombre siguiendo la cadena trófica (Fernandes et al., 2008; Uysal et al., 2008). Es bien sabido que existen zonas marinas en las que los aportes de las aguas desde zonas industriales, urbanas o agrícolas pueden conllevar a un aumento local considerable en los niveles de metales, insecticidas, materia orgánica, etc., pudiendo afectar a las comunidades de animales que se localizan en ellas (Mendil et al., 2010; Sapkota et al., 2008). Por ello, es necesario conocer si estos niveles pueden afectar negativamente a los peces, tanto silvestres como cultivados, ya que serán destinados al consumo humano. Entre los contaminantes en los ecosistemas acuáticos, los metales (As, Cu, Pb, Hg, Cd etc.) están recibiendo cada vez más atención. Entre los efectos adversos de éstos, se describen casos de mortalidad, alteraciones en el metabolismo, desarrollo y maduración sexual, así como en los parámetros hematológicos. Además, se sabe que la exposición a diversos metales afecta negativamente a la respuesta inmunitaria (Bols et al., 2001; Cuesta et al., 2011; Di Giulio y Hinton, 2008; Sweet y Zelikoff, 2001), y por tanto aumenta la incidencia de enfermedades provocando grandes

pérdidas económicas. Por otra parte, la inmensa mayoría de estudios en peces se han centrado en especies de agua dulce (salmónidos y ciprínidos mayoritariamente) y son muchos menos los datos disponibles en especies de agua marina, sobre todo aquellas destinadas al consumo humano, como es el caso de la dorada y la lubina.

En la presente Tesis Doctoral, hemos trabajado con 4 metales no esenciales: cadmio ($CdCl_2$), mercurio [en forma inorgánica ($HgCl_2$) y orgánica (metilmercurio, CH_3HgCl (MeHg)], plomo ($PbNO_3$) y arsénico (As_2O_3). El Cd se utiliza cada vez más en diversas industrias tales como la producción de las baterías de níquel-cadmio y, en menor medida, pigmentos, revestimientos, estabilizadores para plásticos y aleaciones no ferrosas y dispositivos fotovoltaicos (Waisberg et al., 2003). Los principales efectos tóxicos del Cd en los peces se manifiestan en las branquias y el riñón, desencadenando alteraciones osmorreguladoras. Por otra parte, el Cd es un modulador del sistema endocrino e inmunitario, pudiendo afectar a parámetros hematológicos y provocando anemia, alteraciones histológicas y deformidades esqueléticas (Guardiola et al., 2013a; Kennedy, 2011; Voccia et al., 1996; Zelikoff et al., 1995). En el caso del Hg, el uso histórico de éste metal incluye fungicidas, antisépticos tópicos, conservantes de vacunas, desinfectantes, laxantes, diuréticos, aerosoles nasales, pilas, termómetros, cremas para aclarar la piel, cosméticos y otras aplicaciones biomédicas (Clarkson y Magos, 2006). En cuanto a su bioacumulación, las concentraciones de MeHg se magnifican en la cadena alimentaria, alcanzando concentraciones en los peces de 10.000 a 100.000 veces mayor que en el agua circundante (Clarkson y Magos, 2006). Los tejidos diana primarios para el Hg son el sistema nervioso central y los riñones (Clarkson et al., 2003). Además, produce pérdida de apetito, lesiones cerebrales, cataratas, coordinación motora anormal y cambios de comportamiento que conllevan en el caso de los peces a tener problemas de crecimiento, reproducción y desarrollo. En cuanto al Pb, es un contaminante común en el medio natural que puede entrar en la columna de agua a través de la erosión geológica y la acción volcánica, o mediante diversas prácticas antropogénicas incluidas la fusión, la quema de carbón y su uso en la gasolina, baterías y pinturas (ATSDR, 2007). La contaminación del agua por medio de prácticas antropogénicas es la principal causa de envenenamiento por Pb en el pescado (Monteiro et al., 2011; Rogers y Wood, 2004). Además, el Pb puede ser bioconcentrado a partir del agua, pero no biomagnificarse, y las concentraciones tienden a disminuir con el aumento de nivel trófico. El Pb causa cáncer, afecta negativamente a la función del

hígado y el tiroides, y disminuye la resistencia a las enfermedades (ATSDR, 2007). Otros efectos incluyen daño muscular, espinal, degeneración neurológica (Tiffany-Castiglioni, 1993), inhibición del crecimiento, problemas reproductivos y efectos hematológicos tales como la interrupción en la síntesis de la hemoglobina debido a que el Pb inhibe la ALA-deshidratasa, una enzima crítica en la incorporación de hierro en el grupo hemo de los eritrocitos (Alves y Wood, 2006). Por último, el As es un metaloide tóxico que se produce de forma natural en el medio ambiente y se encuentra en el suelo, el aire y el agua (Duker et al., 2005; Huang et al., 2004). Las formas orgánicas que se producen en el medio ambiente se consideran generalmente no tóxicas, mientras que las formas inorgánicas (As^{3+} y As^{5+}) sí son tóxicas (Cervantes et al., 1994). La acumulación de As en el medio ambiente ha incrementado debido a actividades humanas como la quema de combustibles fósiles, la fusión de metales, minería y el uso de semiconductores (Nayak et al., 2007; Smedley y Kinniburgh, 2002). Por otra parte, las fuentes industriales de compuestos inorgánicos de As son insecticidas, herbicidas, fungicidas, pinturas antiincrustantes y conservantes de la madera. El As ambiental se acumula principalmente en la retina, el hígado y el riñón de los peces expuestos. Hay estudios que sugieren que el As induce estrés oxidativo (Bhattacharya y Bhattacharya, 2007), inflamación del hígado (Pedlar et al., 2002), hipertrofia, inducción de proteínas de estrés, apoptosis de los hepatocitos (Datta et al., 2007) y alteraciones del sistema inmunitario (Cordero et al., 2016 submitted; Ghosh et al., 2007; Guardiola et al., 2013b).

Por otro lado, el desarrollo de métodos alternativos a la experimentación animal basados en el modelo de las 3Rs (reducción, refinamiento y reemplazo) ha ocasionado una reducción en el número de animales de laboratorio, cumpliendo así con una mejora de las buenas prácticas éticas en investigación animal. En peces, al igual que en mamíferos, el uso de líneas celulares o cultivos primarios se ha convertido en una herramienta muy útil en el ámbito de la Toxicología para evaluar parámetros celulares alterados tras la exposición a un tóxico, como son la viabilidad, cambios morfológicos, metabolismo, cambios en la permeabilidad de la membrana celular, proliferación o crecimiento (Bols et al., 2005; Fent, 2001). De hecho, muchos ensayos citotóxicos con contaminantes han correlacionado positivamente los valores de las dosis que producen una mortalidad del 50% de la población (DL_{50}) en experimentos llevados a cabo tanto *in vivo* como *in vitro* en peces, demostrando así la gran utilidad de los cultivos celulares de peces en

Toxicología (Castaño et al., 1996; Fent, 2001; Segner, 2004; Vega et al., 1996). Actualmente, hay alrededor de 300 líneas celulares derivadas de peces entre especies de agua dulce (BF-2, WSF, GFM, PSP y más recientemente, líneas celulares derivadas del pez cebra) o marinas, aunque en menor proporción (SAF-1, SPH, GF-1, CRF-1). Igualmente, se han desarrollado cultivos primarios de células de peces a partir de explantes de cerebro, músculo o piel, entre otros (Bols et al., 1994; Minghetti et al., 2014; Naicker et al., 2007). Las ventajas que el uso de líneas celulares o cultivos primarios de peces puede proporcionar son varias: son técnicas fáciles de reproducir, rápidas, con un moderado coste, y como ya hemos comentado anteriormente, sirven para reducir el uso de animales de experimentación (Bols et al., 2005; Fent, 2001). Además, en determinados casos, los cultivos celulares primarios representan modelos más realistas y apropiados de tejidos *in vivo* que las líneas celulares (Freshney, 2011). Por ello, investigaciones sobre los efectos de los metales en especies de peces cultivados y sus líneas celulares o cultivos celulares primarios deben llevarse a cabo, no sólo desde un punto de vista ambiental, sino también teniendo en cuenta los intereses de la industria de la acuicultura y de los consumidores.

Una de las características de los contaminantes, sobre todo de los metales, es que son bioacumulados por los organismos y aumentan su concentración a lo largo de la cadena trófica. Esto es debido, entre otros factores, a la presencia de proteínas celulares de defensa como las metalotioneínas (MT) o las proteínas de choque térmico (HSP). Las metalotioneínas son proteínas ricas en cisteína con una gran afinidad por unir metales como el Cd, Hg o Cu (Bebianno et al., 2007; Roméo et al., 2000; Shaw et al., 1991), por lo que su implicación en la desintoxicación de metales y en el mantenimiento de la homeostasis de iones metálicos esenciales como el Zn ha sido ampliamente estudiado (Klaassen et al., 2009; Templeton et al., 1991). En todos los vertebrados, incluyendo los peces, se han descrito 2 isoformas de metalotioneínas (MT1 y MT2) (Smirnov, 2005; Vasák y Hasler, 2000), cuya presencia se ha descrito en diversos tejidos (Masters et al., 1994; Moffatt et al., 1997). La inducción de MTs no sólo puede aumentar la tolerancia de los peces a los metales, sino también disminuir las tasas de excreción de metal debido a su secuestro, con lo que puede aumentar el potencial de bioconcentración y bioacumulación dentro de un pez y ser transferido a través de una cadena trófica (Kennedy, 2011). Con respecto a las HSP o proteínas del estrés, son chaperonas ampliamente conservadas que ayudan al plegamiento de proteínas recién sintetizadas y

a su transporte celular (Borges y Ramos, 2005; Walter y Butcher, 2002) y cuya función se ha relacionado tanto en mamíferos como en peces con la protección celular, la supervivencia e incluso la respuesta inmunitaria (Aneja et al., 2006; Basu et al., 2001; Fink et al., 1998).

Centrándonos más en los mecanismos toxicológicos que los metales pueden desencadenar a nivel molecular, existe numerosa información en mamíferos que describe cómo los metales producen estrés oxidativo, como resultado de una descompensación entre la producción de ROS y los mecanismos antioxidantes de defensa. Sin embargo, en peces la información es más escasa. Algunos estudios avalan la respuesta oxidativa en peces después de una exposición a contaminantes (Slaninová et al., 2009; Lushchak, 2011), por lo tanto, la evaluación del daño oxidativo y las defensas antioxidantes en los peces puede reflejar la contaminación por metales del medio acuático (Livingstone, 2003). El estrés oxidativo es un inevitable aspecto de la vida aeróbica. Entre los ROS, nos encontramos con el anión superóxido ($O_2^{\cdot-}$), el peróxido de hidrógeno (H_2O_2), el radical hidroxilo ($OH^{\cdot}$), el ácido hipoclorito (HOCl) o el peroxinitrito ($ONNO^-$). El orgánulo celular donde principalmente se producen estos radicales es la mitocondria, en la que el O_2 es el aceptor terminal de electrones en la cadena de transporte de electrones mitocondrial y el cual se reduce a agua generando ROS al mismo tiempo que se produce ATP (Finkel y Holbrook, 2000). Estos radicales de oxígeno también se producen de alguna manera en el retículo endoplasmático, donde se localiza el complejo citocromo P450 (Della-Torre et al., 2012a, b); por ciertas enzimas como las ciclooxigenasas o xantina oxidasas; en los eritrocitos, como consecuencia de la oxidación de la hemoglobina a metahemoglobina (Çimen, 2008; Giulivi y Davies, 2001) y en las células fagocíticas (macrófagos y granulocitos neutrófilos) durante el proceso de explosión respiratoria (DeCoursey, 2010), siendo en estas últimas un mecanismo vital en la eliminación de patógenos.

Una vez producidos dichos ROS, estos deben ser eficazmente eliminados por la maquinaria celular para no resultar tóxicos. Entre estos mecanismos antioxidantes se encuentran las enzimas superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx) o glutatión reductasa (GR), y en menor medida, aunque no menos importantes, las peroxirredoxinas (PRXs) (Di Giulio y Meyer, 2008; Lushchak, 2011). En estudios llevados a cabo en peces, se ha observado un incremento (Bhattacharya y

Bhattacharya, 2007; Guardiola et al., 2016; Rajeshkumar et al., 2013) o un descenso (Altikat et al., 2014; Hsu et al., 2013; Li et al., 2013; Minghetti et al., 2011) de estos sistemas enzimáticos tras la exposición a metales. Aunque existe una cierta controversia en la bibliografía, lo cierto es que estas enzimas se consideran buenos biomarcadores del estrés oxidativo producido por metales en peces (Di Giulio y Meyer, 2008). Además, a las MTs también se les ha atribuido una función antioxidante al ser capaces no sólo de sintetizarse tras una situación de estrés oxidativo, sino que eliminan especies reactivas de oxígeno (Klaassen et al., 2009; Ruttkay et al., 2013) en el llamado ciclo redox de las MTs (Kang, 2006; Schwarz et al., 1995).

Las consecuencias del estrés oxidativo son varias. Entre ellas, la peroxidación lipídica es quizás la más estudiada (Devyatkin et al., 2006) aunque no es la única; proteínas y ADN también sufren daños directos tras la exposición a ROS (Marnett, 2000). Finalmente, si el balance oxidativo supera a los sistemas antioxidantes, en la célula se activan los mecanismos que conducen a una muerte celular. Diversos estudios han demostrado la muerte celular inducida por metales, sin embargo, el mecanismo por el cuál las células mueren, pudiendo ser por apoptosis o por necrosis, es objeto de estudio. La apoptosis o muerte celular programada ocurre bajo condiciones controladas con el fin de eliminar células dañadas durante su desarrollo y crecimiento (Rana, 2008) y está desencadenada por 2 vías, la extrínseca y la intrínseca. En la vía extrínseca, una señal externa o ligando se une a los receptores del factor de necrosis tumoral (TNFR) desencadenando la activación de la caspasa 8, y consecuentemente activación de las caspasas 3 y 7, las caspasas efectoras (Pulido et al., 2003). Sin embargo, la vía intrínseca es activada por factores intracelulares como el estrés oxidativo o daño en el DNA, con la consiguiente desregulación entre las proteínas anti-apoptóticas (Bcl2, Bcl-xL, etc.) y pro-apoptóticas (Bax, Bad, Bak, etc.), lo que conlleva a la despolarización de la membrana mitocondrial, liberación del citocromo C que junto con el factor de activación apoptótico (Apaf-1) activan la cascada de caspasas 9 y 3. Por otro lado, la necrosis es una muerte celular patológica, no regulada, en la que la característica principal es que se produce una rápida pérdida de los potenciales de membrana como consecuencia de una depleción del ATP (López et al., 2003; Yang et al., 2007). Además, la entrada excesiva de Ca^{2+} extracelular o un aumento exacerbado en los niveles de ROS conlleva a la muerte celular por necrosis (Degterev et al., 2005). Sin embargo, en algunos casos se ha descrito un modelo de muerte celular que presenta

simultáneamente características apoptóticas y necróticas (Lee et al., 2006; Sancho et al., 2006; Shih et al., 2003). Así, recientemente se ha descrito un nuevo tipo de muerte celular, llamada necroptosis, y es una forma de muerte celular necrótica inducible que implica ruptura de la membrana celular (Christofferson y Yuan, 2010). A diferencia de la necrosis, se cree que la necroptosis es una muerte celular programada que depende de la activación de TNFR y del receptor de interacción con proteínas 1 (RIP1) (Galluzi y Kroemer et al., 2008; Ofengeim y Yuan, 2013).

Por último, como ya hemos mencionado anteriormente, los metales son capaces de alterar la inmunocompetencia en los peces (Hermann y Kim, 2005). De hecho, se presta cada vez más atención al sistema inmunitario de los peces como un indicador de la exposición a contaminantes ambientales (Zelikoff et al., 1995). Además, la acuicultura y el consumo humano han incentivado aún más el interés sobre el bienestar del pez y sobre su sistema inmunitario. Sin embargo, actualmente se dispone de poca información sobre cómo afectan los tóxicos a la respuesta inmunitaria de peces. Estudios *in vivo* han confirmado que la exposición a metales causa una disminución de la respuesta inmunitaria humoral (niveles de anticuerpos y actividades de lisozima, del complemento, de proteína C reactiva, etc.) y celular (el recuento de leucocitos, la actividad citotóxica mediada por células, la fagocitosis y el estallido respiratorio) de peces, aunque otros estudios han demostrado un aumento o no alteración de las mismas (Bols et al., 2001; Cuesta et al., 2011). Sin embargo, la información procedente de estudios *in vitro* es escasa. En el presente trabajo, proponemos el uso de leucocitos como herramienta y modelo para estudios inmunotoxicológicos *in vitro*. Según nuestro conocimiento, de entre las escasas líneas celulares de peces que derivan de leucocitos o células inmunitarias, no se ha empleado ninguna en estudios de Toxicología, por lo que los escasos estudios se han realizado usando cultivos primarios de leucocitos, cuya viabilidad es limitada. Por ejemplo, en los leucocitos de sangre periférica (PBL) procedentes de la carpa común (*Cyprinus carpio*) expuestos a cromo (Cr) se observó una disminución en la proliferación de linfocitos, así como un descenso en la fagocitosis en el caso de leucocitos procedentes del riñón cefálico (HKLs) en concentraciones que no afectaban a la viabilidad celular (Steinhagen et al., 2004). Además, los PBLs de carpa común expuestos a Cd o Zn mostraron deterioro de la actividad del estallido respiratorio, la cual no fue modificada por Pb o Cu (Wieteska y Wakulska, 2007), mientras que la exposición de los HKLs de carpa a Zn y Mn indujo el estallido

respiratorio y un aumento en la actividad de las células *natural killer* (NK) (Ghanmi et al., 1989). Curiosamente, los leucocitos de lubina expuestos a dosis subletales de Hg redujeron sus funciones fagocitarias (Bennani et al., 1996; Sarmento et al., 2004), mientras que la exposición a Cd desencadenó un incremento de la actividad fagocítica (Bennani et al., 1996). Del mismo modo, muy pocos estudios han contribuido al conocimiento de los efectos de dichos tóxicos en la respuesta inmunitaria a nivel molecular. Así, algunos genes del sistema inmunitario [interleuquina 1β (*il1b*) e *il6*, el factor de necrosis tumoral-alfa (TNFα) o el factor amiloide A sérico (SAA)] sufren una regulación positiva tras estar expuestos a Cu (Teles et al., 2011). Por lo tanto, se necesitan más estudios para aclarar los efectos inmunitarios provocados por los metales en los leucocitos de peces.

En definitiva, en la presente Tesis Doctoral, el uso de líneas celulares o cultivos celulares primarios de peces, de gran interés en acuicultura, como modelos alternativos para estudios toxicológicos ha sido abordado con el fin de esclarecer qué mecanismos están implicados tras la exposición a metales. Concretamente, la viabilidad celular, la producción de radicales libres, los mecanismos de defensa, la muerte celular o los efectos sobre el sistema inmunitario de peces, tanto a nivel funcional como génico, tras la exposición a metales (Cd, Hg, Pb o As) *in vitro* son algunos de los aspectos tratados en el presente trabajo.

2. Objetivos

La presente Tesis Doctoral pretende utilizar líneas celulares y cultivos celulares primarios de dorada (*S. aurata*) y lubina (*D. labrax*) para evaluar el efecto tóxico, y sus mecanismos, de los metales Cd, Hg, Pb y As mediante estudios *in vitro*. Concretamente, los objetivos planteados en el presente trabajo son los siguientes:

1. Generar y caracterizar una nueva línea celular de lubina (DLB-1) además de evaluar su aplicación en toxicología.

2. Estudiar el efecto citotóxico de los metales Cd, Hg, Pb y As sobre líneas celulares (DLB-1 y SAF-1) así como sobre cultivos de eritrocitos circulantes y leucocitos (de sangre y de riñón cefálico) de dorada y de lubina.

3. Estudiar el estrés oxidativo inducido por metales mediante la evaluación de la producción de ROS y la expresión de genes relacionados con la actividad antioxidante.

4. Evaluar, a nivel génico, algunos de los mecanismos de protección celular frente a metales en peces como la expresión de metalotioneínas y proteínas de choque térmico.

5. Analizar los tipos de muerte celular tras la exposición *in vitro* a metales.

6. Determinar el efecto de dichos metales sobre la respuesta inmunitaria de los leucocitos de riñón cefálico de dorada y lubina.

3. Capítulos experimentales

La presente Tesis Doctoral se distribuye en 2 bloques con un total de 6 **capítulos**. En el primer bloque, hemos evaluado los efectos ocasionados tras la exposición a metales como Cd, Hg, Pb, As en dos líneas celulares de peces: la línea celular DLB-1, obtenida y caracterizada para este trabajo, procedente del cerebro de lubina (**capítulo 1**); y la línea celular SAF-1, derivada de fibroblastos de aleta de dorada, la cual es comercial (**capítulo 2**). En el segundo bloque hemos evaluado los efectos citotóxicos de los metales tras 24 h de exposición en cultivos celulares primarios de dorada y lubina. Para ello, hemos aislado eritrocitos (**capítulo 3**) o leucocitos procedentes del riñón cefálico (HKLs) y de sangre (PBLs) (**capítulo 4**), y hemos estudiado la viabilidad y las alteraciones a nivel génico (de genes relacionados con el estrés oxidativo, la muerte celular o el sistema inmunitario) que se producen tras la exposición a los metales. Además, hemos estudiado a nivel funcional y génico el efecto de dichos metales en la respuesta inmunitaria de los leucocitos de riñón cefálico de dorada (**capítulo 5**) y de lubina (**capítulo 6**).

1. En el primer **capítulo**, hemos generado una línea celular (DLB-1) a partir del cerebro de lubina (para la cual no existe ninguna línea celular comercial), la hemos caracterizado y hemos estudiado el efecto de la exposición a metales como el Cd, Hg, Pb o As. Nuestros resultados muestran que se trata de células mayoritariamente con morfología epitelial, con crecimiento rápido, con baja capacidad de transfección y de origen glial, no neuronal. Los ensayos toxicológicos llevados a cabo tras la exposición durante 24 h a los metales mostró que el rojo neutro (RN) fue la técnica colorimétrica más sensible, y que el Cd fue el metal más tóxico, con una DL_{50} igual a 0.004 mM, seguido del Hg, As y Pb. Además, la exposición a Cd o Pb desencadenó una gran producción de ROS en las células DLB-1. Curiosamente, el análisis del ciclo celular de las células DLB-1 expuestas a metales mostró que la exposición a Cd, MeHg o Pb aumentó significativamente el porcentaje de células en la fase G0/G1 mientras que la exposición a Pb y As aumentó el porcentaje de células en fase S y casi eliminó las células en fase G2/M. Por otra parte, todos los metales indujeron muerte celular por apoptosis como se demuestra mediante las técnicas de análisis del ciclo celular y la

unión a Anexina. Estos datos se corroboraron a nivel génico con una inhibición de los genes relacionados con la actividad antioxidante y anti-apoptóticos. Este es el primer estudio en el que la citotoxicidad de metales ha sido evaluada en una línea de células cerebrales de peces y los resultados parecen avalar el uso de las DLB-1 en ensayos toxicológicos.

2. El segundo **capítulo**, describe y compara las herramientas para evaluar los efectos potenciales de contaminación marina sobre la dorada usando la línea celular SAF-1. Para ello, se han evaluado los efectos citotóxicos de los metales Cd, Hg, Pb y As sobre las células SAF-1 tras 24 h de exposición. Para evaluar la viabilidad, se usaron 4 técnicas colorimétricas: el rojo neutro (RN), que tiñe lisosomas; el MTT, que tiñe mitocondrias; la liberación de la lactato deshidrogenasa (LDH), que indica daño en la membrana celular; y el cristal violeta (CV), que tiñe a las proteínas y da idea de las células adheridas y vivas. Los resultados obtenidos mostraron una drástica reducción de la viabilidad tras la exposición a metales, siendo el RN el método más sensible, seguido del MTT, CV y LDH. Además, el MeHg fue el metal más tóxico, con una DL_{50} de 0.01 mM, seguido del As, Cd y Pb. Además, se observó un incremento en los niveles de ROS, sobre todo inducidos por el As, y muerte celular por apoptosis en las células de SAF-1 expuestas a todos los metales. Por otra parte, los correspondientes perfiles de expresión génica apuntan a la inducción del sistema de protección de la *mta*, al estrés oxidativo y a la muerte celular por apoptosis después de la exposición durante 24 h.

3. En el tercer **capítulo**, los eritrocitos de dorada y lubina expuestos durante 24 h a metales mostraron una reducción de la viabilidad de forma dosis-dependiente, siendo el Hg el metal más tóxico, seguido del Cd, el As y el Pb en ambas especies. Además, los niveles de expresión de genes como *mta*, *sod*, *cat*, *prx1*, *gr*, *hsp70* y *90*, *bax* y *calp1* fueron alterados dependiendo del metal expuesto y de la especie. En general, se observó estrés oxidativo, activación de los mecanismos de protección e inducción de la eriptosis o muerte celular programada que tiene lugar en los eritrocitos. Por lo tanto, se confirma el uso de eritrocitos de peces como una herramienta para evaluar el impacto de contaminantes acuáticos, así como su utilidad en el esclarecimiento de los mecanismos toxicológicos de los metales.

4. En el cuarto **capítulo**, leucocitos de riñón cefálico (HKLs) y de sangre (PBLs) de dorada y lubina fueron expuestos a metales durante 24 h. Los resultados muestran que se produce una curva dosis-respuesta tras la exposición a los metales, reduciéndose la viabilidad a medida que aumenta la concentración de éstos. De entre todos los metales, el Hg produjo la mayor toxicidad, seguido del Cd, As y Pb. Interesantemente, los leucocitos de lubina mostraron una mayor resistencia a la exposición de los metales que los de dorada. A su vez, los HKLs fueron más sensibles a los metales mostrando valores de DL_{50} inferiores a los PBLs dentro de la misma especie y mayores alteraciones en la expresión génica. Así, la expresión de genes relacionados con la protección celular (*mta* y *hsp*) o el estrés oxidativo (*sod*, *cat*, *gr*) es disminuida en los HKLs de dorada mientras que aumenta en los PBLs de dorada y en los HKLs y PBLs de lubina. Además, los niveles de expresión de *bax* y *casp3* sugieren una activación de los mecanismos de muerte celular por apoptosis. Finalmente, la expresión de genes relacionados con el sistema inmunitario (*il1b* e *ighm*) se redujo por lo que se podría estar produciendo una inmunosupresión. Este **capítulo** muestra los beneficios de usar leucocitos como biomarcadores de una contaminación por metales, a su vez que aporta luz sobre la regulación a nivel génico.

5. En el quinto **capítulo**, los leucocitos de riñón cefálico (HKLs) de dorada expuestos a los metales durante 30 min o 2 h sufrieron una reducción de la viabilidad dosis-dependiente. Interesantemente, el Hg indujo muerte celular por apoptosis y necrosis simultáneamente, mientras que el Cd, As o Pb sólo indujeron muerte celular por apoptosis. Además, el Cd, Hg, y sobre todo el As, estimularon la producción de ROS en los leucocitos de dorada. Con respecto a las actividades inmunitarias, encontramos diferentes efectos. El Hg y As incrementaron el porcentaje de células fagocíticas (habilidad fagocítica) pero disminuyeron el número de partículas ingeridas por célula (capacidad fagocítica), mientras que el Pb aumentó significativamente los 2 parámetros fagocíticos. De la misma forma, la explosión respiratoria se vió afectada tras la incubación de los leucocitos con los metales, sin embargo, el Pb aumentó la explosión respiratoria. Los resultados en la expresión génica mostraron un aumento del transcrito *sod*, *cat* y *mta* tras la exposición a Cd, sin embargo, Hg y As la inhibieron. En general,

se observa una inmunosupresión de genes relacionados con la respuesta inmunitaria. Nuestros datos sugieren que los mecanismos toxicológicos varían en función del tipo de metal y del tiempo de exposición, pero en general se observa una inmunosupresión del sistema inmunitario de dorada, sobre todo tras la exposición a Hg.

6. En este último **capítulo** de la presente Tesis Doctoral, leucocitos de riñón cefálico de lubina (HKLs) fueron expuestos a metales durante 30 min o 2 h. Los resultados nos mostraron un aumento en la producción de ROS después de la incubación con Cd, Hg y As, sobre todo tras 30 min de exposición. Interesantemente, Hg y Cd indujeron muerte celular por apoptosis o necrosis, mientras que el resto de metales indujeron apoptosis como tipo de muerte celular, en todos los casos con una respuesta dosis-dependiente. Además, la expresión de genes del estrés oxidativo y de apoptosis fue significativamente inducida por Hg y Pb, pero inhibida por As. Con respecto a los genes relacionados con la protección celular, Cd y Pb indujeron la expresión de *mta* y *hsp70* mientras que el Hg la inhibió. En cuanto a las actividades inmunitarias, Cd, Hg y As redujo la habilidad fagocítica mientras que el Pb la incrementó. Sin embargo, todos los metales redujeron la capacidad fagocítica. La explosión respiratoria se vio alterada dependiendo del metal y del tiempo de exposición. En cuanto a la expresión de genes relacionados con el sistema inmunitario, el Hg fue el metal que más alteró dichos tránscritos. Así, la expresión de *ighm* y hepcidina (*hepc*) fue inhibida mientras que los niveles de expresión de la *il1b* se incrementaron significativamente. Este estudio pone de manifiesto nuevamente la aplicación de los leucocitos en ensayos toxicológicos *in vitro.*

4. Conclusiones

1. Hemos generado una línea celular de cerebro de lubina denominada DLB-1 cuyo origen parece glial. Se necesitan más esfuerzos por conseguir líneas celulares de peces para estudios de Biología Celular y Molecular.

2. La exposición a los metales Cd, Hg, As y Pb en las líneas celulares y cultivos celulares primarios de dorada y lubina produjo una reducción en la viabilidad dosis-tiempo dependientes. El MeHg resultó ser el metal más toxico, excepto para la línea DLB-1, mientras que el Pb fue el menos citotóxico.

3. Entre los cultivos celulares primarios, los eritrocitos fueron las células más sensibles a la exposición de metales, seguidos de los leucocitos de riñón cefálico y los leucocitos de sangre.

4. Comparando las dos especies estudiadas, las células de lubina son más resistentes a la exposición por metales que las de dorada.

5. La exposición a metales induce los sistemas de protección celular y antioxidantes, los cuáles son incapaces de compensar la sobreproducción de ROS, desencadenando el estrés oxidativo, el cual conlleva a la muerte celular, principalmente por apoptosis.

6. La respuesta inmunitaria de los leucocitos de riñón cefálico de dorada y lubina se vio disminuida tras la exposición a metales tanto a nivel génico como funcional.

7. Ambas líneas celulares y los cultivos celulares primarios de peces empleados son herramientas valiosas para estudiar los mecanismos de la toxicidad producida por metales.

8. Los eritrocitos de dorada y lubina deberían ser incluidos como modelo *in vitro* en estudios de Toxicología.

References

Adeyemo, O.K., Ajani, F., Adedeji, O.B., Ajiboye, O.O., 2008. Acute toxicity and blood profile of adult *Clarias gariepinus* exposed to lead nitrate. Internet J. Hematol. 4, 1–8.

Aebi, H., 1984. Catalase *in vitro*. Method. Enzymol. 105, 121–126.

AESAN, 2011. Recomendaciones de consumo de pescado (pez espada, tiburón, atún rojo y lucio) debido a la presencia de mercurio. Available online: http://www.aecosan.msssi.gob.es/AECOSAN/web/para_el_consumidor/ampliacion/mercurio_pescado.shtml.

Ahmed, V.P., Chandra, V., Sudhakaran, R., Kumar, S.R., Sarathi, M., Babu, V.S., Ramesh, B., Hameed, A.S., 2009. Development and characterization of cell lines derived from rohu, *Labeo rohita* (Hamilton), and catla, *Catla catla* (Hamilton). J. Fish Dis. 32, 211–218.

Alberts, B., Johnson, A., Lewis, J., 2002. Lymphocytes and the cellular basis of adaptive immunity. Mol. Biol. Cell 4552, 4–8.

Allen, T., Singhal, R., Rana, S.V., 2004. Resistance to oxidative stress in a freshwater fish *Channa punctatus* after exposure to inorganic arsenic. Biol. Trace Elem. Res. 98, 63–72.

Al-Modhefer, A.J.A., Bradbury, M.W.B., Simons, T.J.B., 1991. Observations on the chemical nature of lead in human blood-serum. Clin. Sci. 81, 823–829.

Altikat, S., Uysal, K., Kuru, H.I., Kavasoglu, M., Ozturk, G.N., Kucuk, A., 2014. The effect of arsenic on some antioxidant enzyme activities and lipid peroxidation in various tissues of mirror carp (*Cyprinus carpio carpio*). Environ. Sci. Pollut. Res. Int. 338, 21–24.

Alves, L.C., Wood, C.M., 2006. The chronic effects of dietary lead in freshwater juvenile rainbow trout (*Oncorhynchus mykiss*) fed elevated calcium diets. Aquat. Toxicol. 78, 217–232.

Al-Yakoob, S., Bou-Olayan, A.H., Bahloul, M., 1994. Trace metals in gills of fish from the Arabian Gulf. Bull. Environ. Contam. Toxicol. 53, 718–725.

Amado, L.L., Da-Rosa, C.E., Leite, A.M., Moraes, L., Pires, W.V., 2006. Biomarkers in croakers *Micropogonias furnieri* (Teleostei: Sciaenidae) from polluted and non-polluted areas from the Patos Lagoon estuary (Southern Brazil): evidence of genotoxic and immunological effects. Mar. Pollut. Bull. 52, 199–206.

Aneja, R., Odoms, K., Dunsmore, K., Shanley, P., Wong, H.R., Shanley, T.P., 2006. Extracellular heat shock protein-70 induces endotoxin tolerance in THP-1 cells. J. Immunol. 177, 7184–7192.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.

Ariza, M.E., Bijur, G.N., Williams, M.V., 1998. Lead and mercury mutagenesis: role of H_2O_2, superoxide dismutase, and xanthine oxidase. Environ. Mol. Mutagen. 31, 352–361.

Aschner, M., Onishchenko, N., Ceccatelli, S., 2010. Toxicology of alkylmercury compounds. In: Sigel, A., Sigel, H., Sigel, R.K.O. (Eds). Organometallics in environment and toxicology. RSC Publishing, Cambridge, UK. pp. 403–434.

Ates, B., Orun, I., Talas, Z.S., Durmaz, G., Yilmaz, I., 2008. Effects of sodium selenite on some biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss* Walbbaum 1792) exposed to $Pb^{2+}$and Cu^{2+}. Fish Physiol. Biochem. 34, 53–59.

ATSDR, 2007. Toxicological profile for lead. U.S. Department of health and human services. Public Health Service, Atlanta.

Austin, B., 1999. The effects of pollution on fish health. J. Appl. Microbiol. 85, 234–242.

Ayrton, A., Morgan, P., 2001. Role of transport proteins in drug absorption, distribution and excretion. Xenobiotica 31, 469–497.

Babich, H., Borenfreund, E., 1991. Cytotoxicity and genotoxicity assays with cultured fish cells: a review. Toxicol. In Vitro 5, 91–100.

Babior, B.M., 2000. Phagocytes and oxidative stress. Am. J. Med. 109, 33–44.

Bado-Nilles, A., Quentel, C., Thomas-Guyon, H., Le Floch, S., 2009. Effects of two oils and 16 pure polycyclic aromatic hydrocarbons on plasmatic immune parameters in the European sea bass, *Dicentrarchus labrax* (Linné). Toxicol. In Vitro 23, 235–241.

Bagdonas, E., Vosylienė, M.Z., 2006. A study of toxicity and genotoxicity of copper, zinc and their mixture to rainbow trout (*Oncorhynchus mykiss*). Biologija 1, 8–13.

Bannon, D.I., Olivi, L., Bressler, J., 2000. The role of anion exchange in the uptake of Pb by human erythrocytes and Madin-Darby canine kidney cells. Toxicology 147, 101–107.

Basu, N., Nakano, T., Grau, E.G., Iwama, G.K., 2001. The effects of cortisol on heat shock protein 70 levels in two fish species. Gen. Comp. Endocrinol. 124, 97–105.

Bayen, S., Koroleva, E., Lee, H.K., Obbard, J.P., 2005. Persistent organic pollutants and heavy metals in typical seafoods consumed in Singapore. J. Toxicol. Environ. Health 68, 151–166.

Bayne, C.J., Levy, S., 1991. Modulation of the oxidative burst in trout myeloid cells by adrenocorticotropic hormone and catecholamines: mechanisms of action. J. Leukoc. Biol. 50, 554–560.

Bayoumi, A.E., Pérez-Pertejo, Y., Ordóñez, C., García-Fernández, A.J., Reguera, R.M., Balaña-Fouce, R., Ordóñez, D., 1999. Evaluación de la citotoxicidad de metales *in vitro* en las líneas celulares de peces BF-2 y RTG-2 por dos métodos alternativos. Rev. Toxicol. 16, 17–21.

Beauvais, S.L., Jones, S.B., Parris, J.T., Brewer, S.K., Little, E.E., 2001. Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorhynchus mykiss*). Ecotox. Environ. Safe. 49, 84–90.

Bebianno, M.J., Santos, C., Canário, J., Gouveia, N., Sena-Carvalho, D., Vale, C., 2007. Hg and metallothionein-like proteins in the black scabbardfish *Aphanopus carbo*. Food Chem. Toxicol. 45, 1443–1452.

Bechtel, D.G., Lee, L.E.J., 1994. Effects of aflatoxin B1 in a liver cell line from rainbow trout (*Oncorhynchus mykiss*). Toxicol. In Vitro 8, 317–328.

Begley, T.J., Samson, L.D., 2004. Network responses to DNA damaging agents. DNA Repair 3, 1123–1132.

Béjar, J., Borrego, J.J., Álvarez, M.C., 1997. A continuous cell line from the cultured marine fish. Aquaculture 150, 143–153.

Bell, J.G., McEvoy, J., Tocher, D.R., Sargent, J.R., 2000. Depletion of α-tocopherol and astaxanthin in Atlantic salmon (*Salmo salar*) affects autoxidative defense and fatty acid metabolism. J. Nutr. 130, 1800–1808.

Benedetti, M., Martuccio, G., Fattorini, D., Canapa, A., Barucca, M., Nigro, M., Regoli, F., 2007. Oxidative and modulatory effects of trace metals on metabolism of polycyclic aromatic hydrocarbons in the Antarctic fish *Trematomus bernacchii*. Aquat. Toxicol. 85, 167–175.

Bennani, N., Schmid-Alliana, A., Lafaurie, M., 1996. Immunotoxic effects of copper and cadmium in the sea bass *Dicentrarchus labrax*. Immunopharmacol. Immunotoxicol. 18, 129–144.

Bentley, P.J., 1991. Accumulation of cadmium by channel catfish (*Ictalurus punctatus*): influx from environmental solutions. Comp. Biochem. Physiol. C 99, 527–529.

Betoulle, S., Duchiron, C., Deschaux, P., 2000. Lindane differently modulates intracellular calcium levels in two populations of rainbow trout (*Oncorhynchus mykiss*) immune cells: head kidney phagocytes and peripheral blood leucocytes. Toxicology 145, 203–215.

Bhattacharya, A., Bhattacharya, S., 2007. Induction of oxidative stress by arsenic in *Clarias batrachus*: involvement of peroxisomes. Ecotox. Environ. Safe. 66, 178–187.

Bilmen, S., Aksu, T.A., Gümüşlü, S., Korgun, D.K., Canatan, D., 2001. Antioxidant capacity of G-6-PD-deficient erythrocytes. Clin. Chim. Acta 303, 83–86.

Blanco, F.J., Rego, I., Ruiz-Romero, C., 2011. The role of mitochondria in osteoarthritis. Nat. Rev. Rheumatol. 7, 161–169.

Bloch, S.R., Vo, N.T.K., Walsh, S.K., Chen, C., Lee, L.E.J., Hodson, P.V., Bols, N.C., 2015. Development of a cell line from the American eel brain expressing endothelial cell properties. In Vitro Cell. Dev. Biol. Anim. 52, 395–409.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., Wright, W.E., 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279, 349–352.

Bogé, G., Roche, H., 1996. Cytotoxicity of phenolic compounds on *Dicentrarchus labrax* erythrocytes. Bull. Environ. Contam. Toxicol. 57, 171–178.

Bogé, G., Roche, H., 2004. *In vitro* effects of wastewater treatment plant effluent on sea bass red blood cells. Comp. Biochem. Physiol. C 139, 17–22.

Bols, N.C., Barlian, A., Chirino-Trejo, M., Caldwell, S.J., Goegan, P., Lee, L.E.J., 1994. Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum) gills. J. Fish Dis. 17, 601–611.

Bols, N.C., Brubacher, J.L., Ganassin, R.C., Lee, L.E., 2001. Ecotoxicology and innate immunity in fish. Dev. Comp. Immunol. 25, 853–873.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. In: Moon, T.W., Mommsen, T.P. (Eds). Biochemistry and molecular biology of fishes. Elsevier Science, Amsterdam, pp. 43–84.

Bols, N.C., Lee, L.E.J., 1991. Technology and uses of cell culture from tissues and organs of bony fish. Cytotechnology 6, 163–187.

Borges, J.C., Ramos, C.H., 2005. Protein folding assisted by chaperones. Protein Pept. Lett. 12, 257–261.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Borner, C., 2003. The Bcl-2 protein family : sensors and checkpoints for life-or-death decisions. Mol. Immunol. 39, 615–647.

Boudou, A., Delnomdedieu, M., Georgescauld, D., Ribeyre, F., Saouter, E., 1991. Fundamental roles of biological barriers in mercury accumulation and transfer in freshwater ecosystems (analysis at organism, organ, cell and molecular levels). Water Air Soil Pollut. 56, 807–821.

Bourdineaud, J.P., Baudrimont, M., González, P., Moreau, J.L., 2006. Challenging the model for induction of metallothionein gene expression. Biochimie 88, 1787–1792.

Bradbury, S.P., Carlson, R.W., Henry, T.R., Tala, R.H., Stephanie, P., Cowden, J., 2008. Toxic responses of the fish nervous system. In: Di Giulio, R.T., Hinton, D.E. (Eds). The toxicology of fishes. CRC Press, Florida, USA, pp. 417–455.

Bradman, A., Eskenazi, B., Sutton, P., Athanasoulis, M., Goldman, L.R., 2001. Iron deficiency associated with higher blood lead in children living in contaminated environments. Environ. Health Perspect. 109, 1079–1084.

Brady, T.C., Chang, L.Y., Day, B.J., Crapo, J.D., 1997. Extracellular superoxide dismutase is upregulated with inducible nitric oxide synthase after NF-kappa B activation. Am. J. Physiol. 273, 1002–1006.

Branco, V., Canário, J., Lu, J., Holmgren, A., Carvalho, C., 2012. Mercury and selenium interaction *in vivo*: effects on thioredoxin reductase and glutathione peroxidase. Free Radic. Biol. Med. 52, 781–793.

Bressler, J.P., Olivi, L., Cheong, J.H., Kim, Y., Bannon, D., 2004. Divalent metal transporter 1 in lead and cadmium transport. Ann. N.Y. Acad. Sci. 1012, 142–152.

Brinkmann, M., Blenkle, H., Salowsky, H., Bluhm, K., Schiwy, S., Tiehm, A., Hollert, H., 2014. Genotoxicity of heterocyclic PAHs in the micronucleus assay with the fish liver cell line RTL-W1. PLoS One 9, e85692.

Buikema, A.L., Niederlehner, B.R., Cairns, J.R.J., 1982. Biological monitoring. Part IV. Toxicity testing. Water Res. 16, 239–262.

Buonocore, F., Libertini, A., Prugnoli, D., Mazzini, M., Scapigliati, G., 2006. Production and characterization of a continuous embryonic cell line from sea bass (*Dicentrarchus labrax* L.). Mar. Biotechnol. 8, 80–85.

Bustamante, J., Nutt, L., Orrenius, S., Gogvadze, V., 2005. Arsenic stimulates release of cytochrome c from isolated mitochondria via induction of mitochondrial permeability transition. Toxicol. Appl. Pharmacol. 207, 110–116.

Cai, L., Klein, J.B., Kang, Y.J., 2000. Metallothionein inhibits peroxynitrite-induced DNA and lipoprotein damage. J. Biol. Chem. 275, 38957–38960.

Cai, Y., Ren, X., Xu, D., Wang, M., Wu, X., 2004. Apoptosis induced by cadmium and the expression of *bcl2* and *p53* genes in LLC–P1 cells. Wei Sheng Yan Jiu 33, 663–665.

Calatayud, M., Barrios, J.A., Vélez, D., Devesa, V., 2012. *In vitro* study of transporters involved in intestinal absorption of inorganic arsenic. Chem. Res. Toxicol. 25, 446–453.

Calderón-Salinas, J.V., Quintanar-Escorza, M.A., Hernández-Luna, C.E., González-Martínez, M.T., 1999. Effect of lead on the calcium transport in human erythrocyte. Hum. Exp. Toxicol. 18, 146–153.

Campana, O., Sarasquete, C., Blasco, J., 2003. Effect of lead on ALA-D activity, metallothionein levels, and lipid peroxidation in blood, kidney, and liver of the toadfish *Halobatrachus didactylus*. Ecotox. Environ. Safe. 55, 116–125.

Cao, L., Huang, W., Liu, J., Yin, X., Dou, S., 2010. Accumulation and oxidative stress biomarkers in Japanese flounder larvae and juveniles under chronic cadmium exposure. Comp. Biochem. Physiol. C 151, 386–392.

Carbonell, G., Martínez-Pereda, J., Tarazona, J., 1998. Mobilization of essential metals during and after short-term lethal cadmium exposure in rainbow trout (*Oncorhynchus mykiss*). Ecotoxicol. Environ. Restor. 1, 85–91.

Carey, J.B., Allshire, A., van Pelt, F.N., 2006. Immune modulation by cadmium and lead in the acute reporter antigen-popliteal lymph node assay. Toxicol. Sci. 91, 113–122.

Carlson, E.A., Li, Y., Zelikoff, J.T., 2002. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. Aquat. Toxicol. 56, 289–301.

Carranza-Rosales, P., Said-Fernandez, S., Sepúlveda-Saavedra, J., Cruz-Vega, D.E., Gandolfi, A.J., 2005. Morphologic and functional alterations induced by low doses of mercuric chloride in the kidney OK cell line: ultrastructural evidence for an apoptotic mechanism of damage. Toxicology 210, 111–121.

Carvalho-Neta, R.N.F., Torres, A.R., Abreu-Silva, A.L., 2012. Biomarkers in catfish *Sciades herzbergii* (Teleostei, Aariidae) from polluted and non-polluted areas (São Marcos' Bay, Northeastern Brazil). Appl. Biochem. Biotechnol. 166, 1314–1327.

Carvalho-Neta, R.N.F., Pinheiro-Sousa, D.B., Carvalho-Macêdo, I., Yarbrough, E., da Silva-Almeida., Z., Tchaicka, L., Lislea-Sousa, A., 2015. Genotoxic and hematological parameters in *Colossoma macropomum* (Pisces, Serrasalmidae) as biomarkers for environmental impact assessment in a protected area in northeastern Brazil. Environ. Sci. Pollut. Res. 22, 15994–16003.

Carvan, M., Gallagher, E.P., Goksøyr, A., Mark, E., Larsson, J., 2007. Roundtable discussion: fish models in toxicology. Zebrafish 4, 9–20.

Casani, D., Randelli, E., Costantini, S., Facchinao, A., Zou, J., Martin, S., Secombes, C.J., Scapigliati, G., Buonocore, F., 2009. Molecular characterisation and structural analysis of an interferon homologue in sea bass (*Dicentrarchus labrax* L.). Mol. Immunol. 46, 943–952.

Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Kawahara, K., Lee, L.E.J., Mothersill, C., Pärt, P., Sintes, J.R., Rufli, H., Smith, R., Wood, C., 2003. The use of fish cells in ecotoxicology. Atla 31, 317–351.

Castaño, A., Cantarino, M.J., Castillo, P., Tarazona, J.V., 1996. Correlations between the RTG-2 cytotoxicity test Ec_{50} and *in vivo* Lc_{50} rainbow trout bioassay. Chemosphere 32, 2141–2157.

Castro-González, M.I., Méndez-Armenta, M., 2008. Heavy metals: implications associated to fish consumption. Environ. Toxicol. Pharmacol. 26, 263–271.

Ceccatelli, S.E.D., Moors, M., 2010. Methylmercury-induced neurotoxicity and apoptosis. Chem. Biol. Interact. 188, 301–308.

Celi, M., Vazzana, M., Sanfratello, M.A., Parrinello, N., 2012. Elevated cortisol modulates *hsp70* and *hsp90* gene expression and protein in sea bass head kidney and isolated leukocytes. Gen. Comp. Endocrinol. 175, 424–431.

Cervantes, C., Ji, G., Ramirez, J.L., Silver, S., 1994. Resistance to arsenic compounds in microorganisms. FEMS Microbiol. Rev. 15, 355–367.

Chabrillón, M., Rico, R.M., Arijo, S., Díaz-Rosales, P., Balebona, M.C., Moriñigo, M.A., 2005. Interactions of microorganisms isolated from gilthead sea bream, *Sparus aurata* L., on *Vibrio harveyi*, a pathogen of farmed Senegalese sole, *Solea senegalensis* (Kaup). J. Fish Dis. 28, 531–537.

Chan, K.M., Davies, P.L., Childs, S., Veinot, L., Ling, V., 1992. P-glycoprotein genes in the winter flounder, *Pleuronectes americanus*: isolation of two types of

genomic clones carrying 3´ terminal exons. Biochim. Biophys. Acta 1171, 65–72.

Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59, 527–605.

Chen, F., Lu, Y., Zhang, Z., Vallyathan, V., Ding, M., Castranova, V., Shi, X., 2001. Opposite effect of NF-κB and c-Jun N-terminal kinase on p53–independent GADD45 induction by arsenite. J. Biol. Chem. 276, 11414–11419.

Chen, M.J., Chiou, P.P., Liao, Y.H., Lin, C.M., Chen, T.T., 2010. Development and characterization of five rainbow trout pituitary single-cell clone lines capable of producing pituitary hormones. J. Endocrinol. 205, 69–78.

Cheng, H.Y., Li, P., David, M., Smithgall, T.E., Feng, L., Lieberman, M.W., 2004. Arsenic inhibition of the JAK-STAT pathway. Oncogene 23, 3603–3612.

Chi, S.C., Wu, Y.C., Cheng, T.M., 2005. Persistent infection of betanodavirus in a novel cell line derived from the brain tissue of barramundi *Lates calcarifer*. Dis. Aquat. Organ. 65, 91–98.

Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.Y., 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat. Toxicol. 100, 151–159.

Choi, M.K., Kim, B.H., Chung, Y.Y., Han, M.S., 2002. Cadmium-induced apoptosis in h9c2, a7r5, and c6-glial cell. Bull. Environ. Contam. Toxicol. 69, 335–341.

Christensen, M.M., Ellermann-Eriksen, S., Rungby, J., Mogensen, S.C., 1993. Comparison of the interaction of methyl mercury and mercuric chloride with murine macrophages. Arch. Toxicol. 67, 205–211.

Christofferson, D.E., Yuan, J., 2010. Necroptosis as alternative form of programmed cell death. Curr. Opin. Cell Biol. 22, 263–268.

Çimen, M.Y.B., 2008. Free radical metabolism in human erythrocytes. Clin. Chim. Acta 390, 1–11.

Clarkson, T.W., Magos, L., 2006. The toxicology of mercury and its chemical compounds. Crit. Rev. Toxicol. 36, 609–662.

Clarkson, T.W., Magos, L., Myers, G.J., 2003. The toxicology of mercury-current exposures and clinical manifestations. N. Engl. J. Med. 349, 1731–1737.

Clemedson, C., Ekwall, B., 1999. Overview of the final MEIC results. I. The *in vitro–in vitro* evaluation. Toxicol. In Vitro 13, 657–663.

Colombo, M., Hamelin, C., Kouassi, E., Fournier, M., Bernier, J., 2004. Differential effects of mercury, lead, and cadmium on IL-2 production by Jurkat T cells. Clin. Immunol. 111, 311–322.

Comhaire, S., Blust, R., Van Ginneken, L., Vanderborght, O.L.J., 1994. Cobalt uptake across the gills of the common carp, *Cyprinus carpio*, as a function of calcium concentration in the water of acclimation and exposure. Comp. Biochem. Physiol. C 109, 63–76.

Cordero, H., Morcillo, P., Martínez, S., Meseguer, J., Pérez-Sirvent, C., Chaves-Pozo, E., Martínez-Sánchez, M.J., Cuesta, A., Esteban, M.A., 2016. Inorganic arsenic causes apoptosis cell death and immunotoxicity on European sea bass (*Dicentrarchus labrax*). Submitted.

Cordero, H., Guardiola, F.A., Cuesta, A., Meseguer, J., Esteban, M.A., 2014. Arsenic induced inflammation and apoptosis in liver, head-kidney and skin of gilthead seabream (*Sparus aurata*). Ann. Mar. Biol. Res. 1, 1–5.

Cornelis, M., Dupont, C., Wepierre, J., 1992. Prediction of eye irritancy potential of surfactants by cytotoxicity tests *in vitro* on cultures of human skin fibroblasts and keratinocytes. Toxicol. In Vitro 6, 119–128.

Corniola, R.S., Tassabehji, N.M., Hare, J., Sharma, G., Levenson, C.W., 2008. Zinc deficiency impairs neuronal precursor cell proliferation and induces apoptosis via p53-mediated mechanisms. Brain Res. 1237, 52–61.

Cossins, A.R., Crawford, D.L., 2005. Fish as models for environmental genomics. Nat. Rev. Genet. 6, 324–333.

Costa, J., Reis-Henriques, M.A., Castro, L.F.C., Ferreira, M., 2012. Gene expression analysis of ABC efflux transporters, *cyp1a* and gstα *in* Nile tilapia after exposure to benzo(a)pyrene. Comp. Biochem. Physiol. C 155, 469–482.

Costa, P.M., Lobo, J., Caeiro, S., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., Delvalls, T.A., Costa, M.H., 2008. Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. Mutat. Res. 654, 29–37.

Cotou, E., Henry, M., Rigos, G., Alexis, M., 2009. Cellular responses of the farmed sea bass *Dicentrarchus labrax* to copper oxide antifoulants. 9th Symposium on Oceanography and Fisheries, pp. 1297–1302.

Crago, J., Tran, K., Budicin, A., Schreiber, B., Lavado, R., Schelenk, D., 2015. Exploring the impacts of two separate mixtures of pesticide and surfactants on estrogenic activity in male fathead minnows and rainbow trout. Arch. Environ. Contam. Toxicol. 68, 362–370.

Cuesta, A., Esteban, M.A., Meseguer, J., 1999. Natural cytotoxic activity of gilthead seabream (*Sparus aurata* L.) leucocytes assessment by flow cytometry and microscopy. Vet. Immunol. Immunopathol. 71, 161–171.

Cuesta, A., Meseguer, J., Esteban, M.A., 2011. Immunotoxicological effects of environmental contaminants in teleost fish reared for aquaculture In: Stoytcheva, M. (Ed.). Pesticides in the modern world-risks and benefits. InTech, Rijeka, Croatia, pp. 241–266.

Currie, S., Tufts, B.L., 1997. Synthesis of stress protein 70 (Hsp70) in rainbow trout *Oncorhynchus mykiss* red blood cells. J. Exp. Biol. 200, 607–614.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.

Danchin, E., Vitiello, V., Vienne, A., Richard, O., Gouret, P., McDermott, M.F., Pontarotti, P., 2004. The major histocompatibility complex origin. Immunol.

Rev. 198, 216–232.

Danilova, N., Bussmann, J., Jekosch, K., Steiner, L.A., 2005. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat. Immunol. 6, 295–302.

Das, A.K., Bag, S., Sahu, R., Dua, T.K., Sinha, M.K., Gangopadhyay, M., Zaman, K., Dewanjee, S., 2010. Protective effect of *Corchorus olitorius* leaves on sodium arsenite-induced toxicity in experimental rats. Food Chem. Toxicol. 48, 326–335.

Datta, S., Ghosh, D., Saha, D.R., Bhattacharaya, S., Mazumder, S., 2009. Chronic exposure to low concentration of arsenic is immunotoxic to fish: role of head kidney macrophages as biomarkers of arsenic toxicity to *Clarias batrachus*. Aquat. Toxicol. 92, 86–94.

Datta, S., Saha, D.R., Ghosh, D., Majumdar, T., Bhattacharya, S., Mazumder, S., 2007. 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, 339–349.

Davis, S.R., Cousins, R.J., 2000. Metallothionein expression in animals: a physiological perspective on function. J. Nutr. 130, 1085–1088.

Dayeh, V.R., Lynn, D.H., Bols, N.C., 2005. Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, *Tetrahymena thermophila*. Toxicol. In Vitro 19, 399–410.

De Almeida, C.J.G., Linden, R., 2005. Phagocytosis of apoptotic cells: a matter of balance. Cell. Mol. Life Sci. 62, 1532–1546.

De Coursey, T.E., 2010. Voltage-gated proton channels find their dream job managing the respiratory burst in phagocytes. Physiology 25, 27–40.

De Gennaro, L.D., 2002. Lead and the developing nervous system. Growth Dev. Aging. 66, 43–50.

De Kretser, A.J., Waldron, H.A., 1963. The mechanical fragility of the red cell in patients with lead poisoning. Brit. J. Ind. Med. 20, 31–69.

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., Cuny, G.D., Mitchison, T.J., Moskowitz, M.A., Yuan, J., 2005. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat. Chem. Biol. 1, 112–119.

Dekkers, D.W., Comfurius, P., Bevers, E.M., Zwaal, R.F., 2002. Comparison between Ca^{2+} induced scrambling of various fluorescently labelled lipid analogues in red blood cells. Biochem. J. 362, 741–747.

Della-Torre, C., Tornambè, A., Cappello, S., Mariottini, M., Perra, G., Giuliani, S., Amato, E., Falugi, C., Crisari, A., Yakimov, M.M., Magaletti, E., 2012a. Modulation of CYP1A and genotoxic effects in European seabass (*Dicentrarchus labrax*) exposed to weathered oil: a mesocosm study. Mar. Environ. Res. 76, 48–55.

Della-Torre, C., Zaja, R., Loncar, J., Smital, T., Focardi, S., Corsi, I., 2012b. Interaction of ABC transport proteins with toxic metals at the level of gene and transport activity in the PLHC-1 fish cell line. Chem. Biol. Interact. 198, 9–17.

Devyatkin, A.A., Revin, V.V., Yudanov, M.A., Kozlova, O.V., Samuilov, V.D., 2006. Effect of hydrogen peroxide on ejection of cell nucleus from pigeon erythrocytes and state of membrane lipids. Bull. Exp. Biol. Med. 141, 261–264.

Di Giulio, R.T., Meyer, J.N., 2008. Reactive oxygen species and oxidative stress In: Di Giulio, R.T., Hinton, D.E. (Eds). The toxicology of fishes. CRC Press, Florida, USA, pp. 273–324.

Diaz de Cerio, O., Bilbao, E., Cajaraville, M.P., Cancio, I., 2012. Regulation of xenobiotic transporter genes in liver and brain of juvenile thicklip greymullets (*Chelon labrosus*) after exposure to Prestige-like fuel oil and to perfluorooctane sulfonate. Gene 498, 50–58.

Dietert, R.R., Piepenbrink, M.S., 2006. Lead and immune function. Crit. Rev. Toxicol. 36, 359–385.

Ding, J., Lu, G., Liang, Y., 2014. Evaluation of the interactive effects of lead, zinc and benzo(k)fluoranthene on crucian carp, *Carassius carassius*, using a multiple biomarker approach. Bull. Environ. Contam. Toxicol. 92, 534–539.

Dobrovolskaia, M.A., Germolec, D.R., Weaver, J.L., 2009. Evaluation of nanoparticle immunotoxicity. Nat. Nanotechnol. 4, 411–414.

Doke, S.K., Dhawale, S.C., 2015. Alternatives to animal testing: a review. Saudi Pharm. J. 23, 223–229.

Dorval, J., Hontela, A., 2003. Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Toxicol. Appl. Pharmacol. 192, 191–200.

Drastichova, J., Svobodova, Z., Luskova, V., Machova, J., 2004. Effect of cadmium on hematological indices of common carp (*Cyprinus carpio* L.). Bull. Environ. Contam. Toxicol. 72, 725–732.

Drobná, Z., Walton, F.S., Paul, D.S., Xing, W., Thomas, D.J., Styblo, M., 2010. Metabolism of arsenic in human liver: the role of membrane transporters. Arch. Toxicol. 84, 3–16.

Dubuisson, M., Vander-Stricht, D., Clippe, A., Etienne, F., Nauser, T., Kissner, R., Koppenol, W.H., Rees, J.F., Knoops, B., 2004. Human peroxiredoxin 5 is a peroxynitrite reductase. FEBS Lett. 571, 161–165.

Duffus, J.H, 2002. " Heavy metals "— a meaningless term? (IUPAC Technical Report). Pure Appl. Chem. 74, 793–807.

Duker, A.A., Carranza, E.J.M., Hale, M., 2005. Arsenic geochemistry and health. Environ. Int. 31, 631–641.

Dumaswala, U.J., Zhuo, L., Jacobsen, D.W., Jain, S.K., Sukalski, K.A., 1999. Protein and lipid oxidation of banked human erythrocytes: role of glutathione. Free Radic. Biol. Med. 27, 1041–1049.

Dunier, M., Siwicki, A.K., 1994. Study of the effects of pollutants on fish defence mechanisms. I. *In vitro* influence of heave metals on the spleen and kidney lymphocytes and macrophages activity in carp (*Cyprinus carpio*). Arch. Pol. Fish. 2, 55–66.

Dutsch-Wicherek, M., 2008. The possible biological role of metallothionein in apoptosis. Front. Biosci. 1, 4029–4038.

Dwivedi, N., Flora, S.J.S., 2014. Sub-chronic exposure to arsenic and dichlorvos on erythrocyte antioxidant defense systems and lipid peroxidation in rats. J. Environ. Biol. 36, 383–391.

Ebany, J.M.F., Chakraborty, S., Fretham, S.J.B., Aschner, M., 2012. Cellular transport and homeostasis of essential and nonessential metals. Metallomics 4, 593–605.

EFSA, 2005. Opinion of the scientific panel on contaminants in the food chain on a request from the European Parliament related to the safety assessment of wild and farmed fish. EFSA J. 236, 1–118.

EFSA, 2012. Scientific opinion on the risk for public health related to the presence of mercury and methylmercury in food. EFSA J. 10, 1–241.

Eisele, K., Lang, P.A., Kempe, D.S., Klarl, B.A., Niemöller, O., Wieder, T., Huber, S.M., Duranton, C., Lang, F., 2006. Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. Toxicol. Appl. Pharmacol. 210, 116–122.

Elia, A.C., Galarini, R., Taticchi, M.I., Dorr, A.J.M., Mantilacci, L., 2003. Antioxidant responses and bioaccumulation in *Ictalurus meias* under mercury exposure. Ecotox. Environ. Safe. 55, 162–167.

Ellis, A.E., 2001. Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immunol. 25, 827–839.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem. 1, 529–539.

Erickson, R.J., Nichols, J.W., Cook, P.M., Ankley, G.T., 2008. Bioavailability of chemical contaminants in aquatic systems. In: Di Giulio, R.T., Hinton, D.E. (Eds).The toxicology of fishes. CRC Press, Florida, USA, pp. 9–45.

Esteban, M.A., Chaves-Pozo, E., Arizcun, M., Meseguer, J., Cuesta, A., 2013. Regulation of natural killer enhancing factor (NKEF) genes in teleost fish, gilthead seabream and European sea bass. Mol. Immunol. 55, 275–282.

Esteban, M.A., Mulero, V., Muñoz, J., Meseguer, J., 1998. Methodological aspects of assessing phagocytosis of *Vibrio anguillarum* by leucocytes of gilthead seabream (*Sparus aurata* L.) by flow cytometry and electron microscopy. Cell Tissue Res. 293, 133–141.

FAO, 2012. El estado mundial de la pesca y la acuicultura. Parte I, FAO. ISBN 978-92-5-107225-7.

Farina, M., Aschner, M., Rocha, J.B.T., 2011. Oxidative stress in MeHg-induced neurotoxicity. Toxicol. Appl. Pharmacol. 256, 405–417.

Farrer, D.G., Hueber, S.M., McCabe, M.J., 2005. Lead enhances CD4+ T cell proliferation indirectly by targeting antigen presenting cells and modulating antigen-specific interactions. Toxicol. Appl. Pharmacol. 207, 125–137.

Fatima, M., Ahmad, I., Sayeed, I., Athar, M., Raisuddin, S., 2000. Pollutant-induced over-activation of phagocytes is concomitantly associated with peroxidative

damage in fish tissues. Aquat. Toxicol. 49, 243–250.

Fatima, M., Usmani, N., Firdaus, F., Zafeer, M.F., Ahmad, S.K., Akhtar, K., Dawar, S.M., Husain, M.H., Ahmad, E., Anis, M., Mobarak, H., 2015. *In vivo* induction of anti-oxidant response and oxidative stress associated with genotoxicity and his-topathological alteration in two commercial fish species due to heavy metals exposure in northern India (Kali) river. Comp. Biochem. Physiol. C 176–177, 17–30.

Fedeli, D., Carloni, M., Falcioni, G., 2010. Oxidative damage in trout erythrocyte in response to "*in vitro*" copper exposure. Mar. Environ. Res. 69, 172–177.

Fent, K., 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol. In Vitro 15, 477–488.

Ferencz, Á., Hermesz, E., 2015. Impact of acute Cd^{2+} exposure on the antioxidant defence systems in the skin and red blood cells of common carp (*Cyprinus carpio*). Environ. Sci. Pollut. Res. Int. 22, 6912–6919.

Fernandes, D., Zanuy, S., Bebianno, M.J., Porte, C., 2008. Chemical and biochemical tools to assess pollution exposure in cultured fish. Environ. Pollut. 152, 138–146.

Fernandes, J.M.O., Smith, V.J., 2004. Partial purification of antibacterial proteinaceous factors from erythrocytes of *Oncorhynchus mykiss*. Fish Shellfish Immunol. 16, 1–9.

Fernández, D., García-Gómez, C., Babín, M., 2013. *In vitro* evaluation of cellular responses induced by ZnO nanoparticles, zinc ions and bulk ZnO in fish cells. Sci. Total Environ. 452–453, 262–274.

Fernández-Cruz, M.L., Lammel, T., Connolly, M., Conde, E., Barrado, A.I., Derick, S., Perez, Y., Fernandez, M., Furger, C., Navas, J.M., 2012. Comparative cytotoxicity induced by bulk and nanoparticulated ZnO in the fish and human hepatoma cell lines PLHC–1 and Hep G2. Nanotoxicology 7, 1–18.

Fernández-Trujillo, M.A., Novel, P., Manchado, M., Sepulcre, M.P., Mulero, V., Borrego, J.J., Álvarez, M.C., Béjar, J., 2011. Three Mx genes with differential response to VNNV infection have been identified in Gilthead seabream (*Sparus aurata*). Mol. Immunol. 48, 1216–1223.

Ferraro, M.V.M., Fenocchio, A.S., Mantovani, M.S., Ribeiro, C.O., Cestari, M.M., 2004. Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. Genet. Mol. Biol. 27, 103–107.

Ferreira, M., Santos, P., Rey-Salgueiro, L., Zaja, R., Reis-Henriques, M.A., Smital, T., 2014. The first demonstration of *cyp1a* and the *abc* protein(s) gene expression and activity in european seabass (*Dicentrarchus labrax*) primary hepatocytes. Chemosphere 100, 152–159.

Fink, A.L., 1999. Chaperone-mediated protein folding. Physiol. Rev. 79, 425–449.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.

Firat, O., Kargin, F., 2010. Effects of zinc and cadmium on erythrocyte antioxidant systems of a freshwater fish *Oreochromis niloticus*. J. Biochem. Mol. Toxicol. 24, 223–229.

Fischer, S., Klüver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A.M., Wellner, P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. BMC Biol. 17, 11–69.

Fischer, S., Pietsch, M., Schirmer, K., Luckenbach, T., 2010. Identification of multi-drug resistance associated proteins MRP1 (ABCC1) and MRP3 (ABCC3) from rainbow trout (*Oncorhynchus mykiss*). Mar. Environ. Res. 69, 7–10.

Fischer, U., Utke, K., Somamoto, T., Köllner, B., Ototake, M., Nakanishi, T., 2006. Cytotoxic activities of fish leucocytes. Fish Shellfish Immunol. 20, 209–226.

Fletcher, T.C., 1986. Modulation of nonspecific host defenses in fish. Vet. Immunol. Immunopathol. 12, 59–67.

Föller, M., Braun, M., Qadri, S.M., Lang, E., Mahmud, H., Lang, F., 2010. Temperature sensitivity of suicidal erythrocyte death. Eur. J. Clin. Invest. 40, 534–540.

Föller, M., Kasinathan, R.S., Koka, S., Lang, C., Shumilina, E., Birnbaumer, L., Lang, F., Huber, S.M., 2008. TRPC6 contributes to the $Ca(^{2+})$ leak of human erythrocytes. Cell. Physiol. Biochem. 21, 183–192.

Fotakis, G., Timbrell, J.A., 2006. *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett. 160, 171–177.

Foulkes, E.C., 2000. Transport of toxic heavy metals across cell membranes. Proc. Soc. Exp. Biol. Med. 223, 234–240.

Frerichs, G.N., Rodger, H.D., Peric, Z., 1996. Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. J. Gen. Virol. 77, 2067–2071.

Freshney, R.I., 2011. Culture of animal cells: a manual of basic technique and specialized applications. John Wiley and Sons. Hoboken, New Yersey.

Fu, X., Li, N., Lai, Y., Wang, Y., Shi, C., Huang, Z., Wu, S., 2015. A novel fish cell line derived from the brain of Chinese perch *Siniperca chuatsi*: development and characterization. J. Fish Biol. 86, 32–45.

Fulladosa, E., Deane, E., Ng, A.H., Woo, N.Y., Murat, J.C., Villaescusa, I., 2006. Stress proteins induced by exposure to sublethal levels of heavy metals in sea bream (*Sparus sarba*) blood cells. Toxicol. In Vitro 20, 96–100.

Gagné, F., Fortier, M., Yu, L., Osachoff, H.L., Skirrow, R.C., van Aggelen, G., Gagnon, C., Fournier, M., 2010. Immunocompetence and alterations in hepatic

gene expression in rainbow trout exposed to CdS/CdTe quantum dots. J. Environ. Monit. 12, 1556–1566.

Gallagher, E.P., Di Giulio, R.T., 1992. A comparison of glutathione-dependent enzymes in liver, gills and posterior kidney of channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol. C 102, 543–547.

Galluzzi, L., Kroemer, G., 2008. Necroptosis: a specialized pathway of programmed necrosis. Cell 135, 1161–1163.

García-Santos, S., Vargas-Chacoff, L., Ruiz-Jarabo, I., Varela, J.L., Mancera, J.M., Fontaínhas-Fernandes, A., Wilson, J.M., 2011. Metabolic and osmoregulatory changes and cell proliferation in gilthead sea bream (*Sparus aurata*) exposed to cadmium. Ecotox. Environ. Safe. 74, 270–278.

Ghanmi, Z., Rouabhia, M., Othmane, O., Deschaux, P.A., 1989. Effects of metal ions on cyprinid fish immune response: *in vitro* effects of Zn^{2+} and Mn^{2+} on the mitogenic response of carp pronephros lymphocytes. Ecotox. Environ. Safe. 17, 183–189.

Ghazaly, K.S., 1991. Influences of thiamin on lead intoxication, lead deposition in tissues and lead hematological responses of *Tilapia zillii*. Comp. Biochem. Physiol. C 100, 417–421.

Ghedira, J., Jebali, J., Bouraoui, Z., Banni, M., Guerbej, H., Boussetta, H., 2010. Metallothionein and metal levels in liver, gills and kidney of *Sparus aurata* exposed to sublethal doses of cadmium and copper. Fish Physiol. Biochem. 36, 101–107.

Ghosh, D., Bhattacharya, S., Mazumder, S., 2006. Perturbations in the cat fish immune response by arsenic: organ and cell specific effects. Comp. Biochem. Physiol. C 143, 455–463.

Ghosh, D., Datta, S., Bhattacharya, S., Mazumder, S., 2007. Long-term exposure to arsenic affects head kidney and impairs humoral immune responses of *Clarias batrachus*. Aquat. Toxicol. 81, 79–89.

Ghosh, S., Bhaattacharya, S., 1992. Elevation of C-reactive protein in serum of *Chana punctatus* as an indicator of water pollution. Indian J. Exp. Biol. 30, 736–737.

Giari, L., Manera, M., Simoni, E., Dezfuli, B.S., 2007. Cellular alterations in different organs of European sea bass *Dicentrarchus labrax* (L.) exposed to cadmium. Chemosphere 67, 1171–1181.

Giari, L., Simoni, E., Manera, M., Dezfuli, B.S., 2008. Histo-cytological responses of *Dicentrarchus labrax* (L.) following mercury exposure. Ecotox. Environ. Safe. 70, 400–410.

Gibbs, P.J., Miskiewicz, A.G., 1995. Heavy metals in fish near a major primary treatment sewage plant outfall. Mar. Pollut. Bull. 30, 667–674.

Giblin, F.J., Massaro, E.J., 1975. The erythrocyte transport and transfer of methylmercury to the tissues of the rainbow trout (*Salmo gairdneri*). Toxicology 5, 243–254.

Gillies, R.J., Didier, N., Denton, M., 1986. Determination of cell number in monolayer cultures. Anal. Biochem. 159, 109–113.

Giudice, G., Sconzo, G, Roccheri, M.C., 1999. Studies on heat shock proteins in sea urchin development. Dev. Growth Differ. 41, 375–380.

Giulivi, C., Davies, K.J.A., 2001. Mechanism of the formation and proteolytic release of H_2O_2-induced dityrosine and tyrosine oxidation products in hemoglobin and red blood cells. J. Biol. Chem. 276, 24129–24136.

Goeptar, A.R., Scheerens, H., Vermeulen, N.P.E., 1995. Oxygen reductase and substrate reductase activity of cytochrome P450. Crit. Rev. Toxicol. 25, 25–65.

Goering, P.L., Kuester, R.K., Neale, A.R., Chapekar, M.S., Zaremba, T.G., Gordon, E.A., Hitchins, V.M., 2000. Effects of particulate and soluble cadmium species on biochemical and functional parameters in cultured murine macrophages. In Vitro Mol. Toxicol. 13, 125–136.

Gómez-Sintes, R., Hernández, F., Lucas, J.J., Avila, J., 2011. GSK-3 mouse models to

study neuronal apoptosis and neurodegeneration. Front. Mol. Neurosci. 16, 4–45.

Goyer, R.A., Clarkson, T.W., 1996. Toxic effects of metals. In: Klaassen, C.D. (Ed). Casarett & Doull's toxicology: the basic science of poisons, 5th edn. New York, pp. 811–867.

Griffith, O.W., Bridges, R.J., Meister, A., 1978. Evidence that the gamma-glutamyl cycle functions *in vivo* using intracellular glutathione: effects of amino acids and selective inhibition of enzymes. Proc. Natl. Acad. Sci. USA. 75, 5405–5408.

Guardiola, F.A., Chaves-Pozo, E., Espinosa, C., Romero, D., Meseguer, J., Cuesta, A., Esteban, M.A., 2016. Mercury accumulation, structural damages, and antioxidant and immune status changes in the gilthead seabream (*Sparus aurata* L.) exposed to methylmercury. Arch. Environ. Contam. Toxicol. doi: 10.1007/s00244-016-0268-6.

Guardiola, F.A., Cuesta, A., Meseguer, J., Martínez, S., Martínez-Sánchez, M.J., Pérez-Sirvent, C., Esteban, M.A., 2013a. Accumulation, histopathology and immunotoxicological effects of waterborne cadmium on gilthead seabream (*Sparus aurata*). Fish Shellfish Immunol. 35, 792–800.

Guardiola, F.A., González-Párraga, M.P., Cuesta, A., Meseguer, J., Martínez, S., Martínez–Sánchez, M.J., Pérez–Sirvent, C., Esteban, M.A., 2013b. Immunotoxicological effects of inorganic arsenic on gilthead seabream (*Sparus aurata* L.). Aquat. Toxicol. 134–35, 112–119.

Gwozdzinski, K., Roche, H., Peres, G., 1992. The comparison of the effects of heavy metal ions on the antioxidant enzyme-activities in human and fish *Dicentrarchus labrax* erythrocytes. Comp. Biochem. Phsyiol. C 102, 57–60.

Halliwell, B., Gutteridge, J.M.C., 2015. Free radicals in biology and medicine, 5th ed. Oxford, Oxford University Press.

Hampton, M.B., Kettle, A.J., Winterbourn, C.C., 1998. Inside the neutrophil

phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92, 3007–3017.

Hanschmann, E.M., Godoy, J.R., Berndt, C., Hudemann, C., Lillig, C.H., 2013. Thioredoxins, glutaredoxins, and peroxiredoxins molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling. Antioxid. Redox Signal. 19, 1539–1605.

Hansen, J.D., La Patra, S., 2002. Induction of the rainbow trout MHC class I pathway during acute IHNV infection. Immunogenetics 54, 654–661.

Hansen, J.D., Landis, E.D., Phillips, R.B., 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. USA. 102, 6919–6924.

Hart, W.B., Doudoroff, P., Greenbank, J., 1945. The evaluation of the toxicity of industrial wastes, chemicals, and other substances to freshwater fishes. In: Hart, W.B. (Ed). Manual on Industrial Water and Industrial Water. Philadelphia, pp. 1345–1359.

Hasoon, M.F., Daud, H.M., Abdullah, A., Arshad, S.S., Bejo, H.M., 2011. Development and partial characterization of new marine cell line from brain of Asian sea bass *Lates calcarifer* for virus isolation. In Vitro Cell. Dev. Biol. Anim. 47, 16–25.

He, L., Poblenz, A.T., Medrano, C.J., Fox, D.A., 2000. Lead and calcium produce rod photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore. J. Biol. Chem. 275, 12175–12184.

Hemdan, N.Y.A., Emmrich, F., Adham, K., Wichmann, G., Lehmann, I., El-Massry, E., Ghoneim, H., Lehmann, J., Sack, U., 2005. Dose-dependent modulation of the *in vitro* cytokine production of human immune competent cells by lead salts. Toxicol. Sci. 86, 75–83.

Henderson, L.M., Chappell, J.B., 1993. Dihydrorhodamine 123: a fluorescent probe for superoxide generation?. Eur. J. Biochem. 217, 973–980.

Hermann, A.C., Kim, C.H., 2005. Effects of arsenic on zebrafish innate immune system. Mar. Biotechnol. 7, 494–505.

Hernández-García, A., Romero, D., Gómez-Ramírez, P., María-Mojica, P., Martínez-López, E., García-Fernández, A.J., 2014. *In vitro* evaluation of cell death induced by cadmium, lead and their binary mixtures on erythrocytes of Common buzzard (*Buteo buteo*). Toxicol. In Vitro 28, 300–306.

Higuchi, Y., Yoshimoto, T., 2002. Arachidonic acid converts the glutathione depletion-induced apoptosis to necrosis by promoting lipid peroxidation and reducing caspase-3 activity in rat glioma cells. Arch. Biochem. Biophys. 400, 133–140.

Himeno, S., Takahiro, Y., Hitomi, F., 2009. The role of zinc transporters in cadmium and manganese transport in mammalian cells. Biochimie 91, 1218–1222.

Hinsch, K., Zupanc, G.K.H., 2006. Isolation, cultivation, and differentiation of neural stem cells from adult fish brain. J. Neurosci. Methods 158, 75–88.

Hitomi, J., Christofferson, D.E., Ng, A., Yao, J., Degterev, A., Xavier, R.J., Yuan, J., 2008. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135, 1311–1323.

Hogstrand, C., Wilson, R.W., Polgar, D., Wood, C.M., 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. J. Exp. Biol. 186, 55–73.

Hong, J.Y., Lebofsky, M., Farhood, A., Jaeschke, H., 2009. Oxidant stress-induced liver injury *in vivo*: role of apoptosis, oncotic necrosis, and c-Jun NH2-terminal kinase activation. Am. J. Physiol. Gastrointest. Liver Physiol. 296, 572–581.

Horwich, A.L., 2014. Molecular chaperones in cellular protein folding: The birth of a field. Cell 157, 285–288.

Hsu, T., Huang, K.M., Tsai, H.T., Sung, S.T., Ho, T.N., 2013. Cadmium (Cd)-induced oxidative stress down-regulates the gene expression of DNA mismatch recognition proteins MutS homolog 2 (MSH2) and MSH6 in zebrafish (*Danio rerio*) embryos. Aquat. Toxicol. 126, 9–16.

Huang, C., Ke, Q., Costa, M., Shi, X., 2004. Molecular mechanisms of arsenic carcinogenesis. Mol. Cell. Biochem. 255, 57–66.

Huang, S.S.Y., Strathe, A.B., Fadel, J.G., Lin, P., Liu, T.Y., Hung, S.S.O., 2012. Absorption, distribution, and elimination of graded oral doses of methylmercury in juvenile white sturgeon. Aquat. Toxicol. 122–123, 163–171.

Hunaiti, A., Soud, M., Khalil, A., 1995. Lead concentration and the level of glutathione, glutathione S-transferase, reductase and peroxidase in the blood of some occupational workers from Irbid City, Jordan. Sci. Total Environ. 170, 95–100.

Hunaiti, A., Soud, M., 2000. Effect of lead concentration on the level of glutathione, glutathione S-transferase, reductase and peroxidase in human blood. Sci. Total Environ. 248, 45–50.

Irato, P., Santovito, G., Piccinni, E., Albergoni, V., 2001. Oxidative burst and metallothionein as a scavenger in macrophages. Immunol. Cell. Biol. 79, 251–254.

Iwama, G.K., Vijayan, M.M., Forsyth, R.B., Ackerman, P.A., 1999. Heat shock proteins and physiological stress in fish. Am. Zool. 39, 901–909.

Jan, M., Frantisek, N., 2000. Cadmium-induced changes in cation-osmotic haemolysis in rats. Environ. Toxicol. Pharmacol. 8, 79–81.

Jebali, J., Banni, M., Guerbej, H., Almeida, E.A., Bannaoui, A., Boussetta, H., 2006. Effects of malathion and cadmium on acetylcholinesterase activity and metallothionein levels in the fish *Seriola dumerilli*. Fish Physiol. Biochem. 32, 93–98.

Jia, X., Zhang, H., Liu, X., 2011. Low levels of cadmium exposure induce DNA damage and oxidative stress in the liver of Oujiang colored common carp *Cyprinus carpio* var. color. Fish Physiol. Biochem. 37, 97–103.

Jolly, S., Bado-Nilles, A., Lamand, F., Turies, C., Chadili, E., Porcher, J.M., Betoulle, S., Sánchez, W., 2014. Applications in environmental risk assessment of leucocyte apoptosis, necrosis and respiratory burst analysis on the European bullhead, *Cottus sp*. Environ. Pollut. 184, 9–17.

Jolly, S., Bado-Nilles, A., Lamand, F., Turies, C., Chadili, E., Porcher, J.M. Betoulle, S. Sánchez, W., 2012. Multi-biomarker approach in wild European bullhead, *Cottus* sp., exposed to agricultural and urban environmental pressures: practical recommendations for experimental design. Chemosphere 87, 675–683.

Julliard, A.K., Saucier, D., Astic, L., 1996. Time-course of apoptosis in the olfactory epithelium of rainbow trout exposed to a low copper level. Tissue Cell 28, 367–377.

Kang, Y.J., 2006. Metallothionein redox cycle and function. Exp. Biol. Med. 231, 1459–1467.

Kasten-Jolly, J., Lawrence, D.A., 2014. Lead modulation of macrophages causes multiorgan detrimental health effects. J. Biochem. Mol. Toxicol. 28, 355–372.

Kempe, D.S., Lang, P.A., Eisele, K., Klarl, B.A., Wieder, T., Huber, S.M., Duranton, C., Lang, F., 2005. Stimulation of erythrocyte phosphatidylserine exposure by lead ions. Cell. Physiol. Biochem. 18, 151–154.

Kennedy, C.J., 2011. Toxicology: the toxicology of metals in fishes. In: Farrell, A.P. (Ed). Encyclopaedia of fish physiology: from genome to environment. Academic Press, San Diego, California, USA, pp. 2061–2068.

Kerper, L.E., Ballatori, N., Clarkson, T.W., 1992. Methylmercury transport across the blood-brain barrier by an amino acid carrier. Am. J. Physiol. 262, 761–765.

Kessabi, K., Hwas, Z., Sassi, A., Said, K., Messaoudi, I., 2014. Heavy metal accumulation and histomorphological alterations in *Aphanius fasciatus* (*Pisces, Cyprinodontidae*) from the Gulf of Gabes (Tunisia). Environ. Sci. Pollut. Res. 21, 14099–14109.

Kibria, G., 2014. Trace / heavy metals and its impact on environment, biodiversity and human health: a short review. Research gate online. 1–3. doi:10.13140/RG.2.1.3102.2568.

Kim, A.T., Sarafian, T.A., Shau, H., 1997. Characterization of antioxidant properties of natural killer-enhancing factor-B and induction of its expression by hydrogen peroxide. Toxicol. Appl. Pharmacol. 147, 135–142.

Kim, J.S., Ahn, T., Yim, S.K., Yun, C.H., 2002. Differential effect of copper (II) on the cytochrome P450 enzymes and NADPH-cytochrome P450 reductase: inhibition of cytochrome P450-catalyzed reactions by copper (II) ion. Biochemistry 41, 9438–9447.

Kim, K., Kim, I.H., Lee, K.Y., Rhee, S.G., Stadtman, E.R., 1988. The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O2 mixed-function oxidation system. J. Biol. Chem. 263, 4704–4711.

Kim, S.G., Jee, J.H., Kang, J.C., 2004. Cadmium accumulation and elimination in tissues of juvenile olive flounder, *Paralichthy solivaceus* after subchronic cadmium exposure. Environ. Pollut. 127, 117–123.

Kim, S.H., Sharma, R.P., 2003. Cytotoxicity of inorganic mercury in murine T and B lymphoma cell lines : involvement of reactive oxygen species, Ca^{2+} homeostasis and cytokine gene expression. Toxicol. In Vitro 17, 385–395.

Kim, S.H., Sharma, R.P., 2004. Mercury-induced apoptosis and necrosis in murine macrophages: role of calcium–induced reactive oxygen species and p38 mitogen–activated protein kinase signaling. Toxicol. Appl. Pharmacol. 196, 47–57.

Kim, W.K., Park, J.W., Lim, E.S., Lee, S.K., Kim, J., Kim, S., Lee, S.W., Choi, K., Jung, J., 2014. Tissue-specific antioxidant responses in Pale Chub (*Zacco platypus*) exposed to copper and benzo[a]pyrene. Bull. Environ. Contam. Toxicol. 92, 540–545.

Kirkman, H.N., Galiano, S., Gaetani, G.F., 1987. The function of catalase-bound NADPH. J. Biol. Chem. 262, 660–666.

Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu. Rev. Pharmacol. Toxicol. 39, 267–294.

Klaassen, C.D., Liu, J., Diwan, B.A., 2009. Metallothionein protection of cadmium toxicity. Toxicol. Appl. Pharmacol. 238, 215–220.

Klarl, B.A., Lang, P.A., Kempe, D.S., Niemoeller, O.M., Akel, A., Sobiesiak, M., Eisele, K., Podolski, M., Huber, S.M., Wieder, T., Lang, F., 2006. Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. Am. J. Physiol. Cell Physiol. 290, 244–253.

Kleinow, K.M., Nichols, J.W., Hayton, W.L., McKim, J.M., Barron, M.G., 2008. Toxicokinetics in fishes. In: Di Giulio, R.T., Hinton, D.E. (Eds). The toxicology of fishes. CRC Press, Florida, USA, pp. 55–152.

Komjarova, I., Blust, R., 2009. Effects of Na, Ca, and pH on the simultaneous uptake of Cd, Cu, Ni, Pb, and Zn in the zebrafish *Danio rerio*: a stable isotope experiment. Environ. Sci. Technol. 43, 7958–7963.

Kondera, E., Witeska, M., 2013. Cadmium and copper reduce hematopoietic potential in common carp (*Cyprinus carpio* L.) head kidney. Fish Physiol. Biochem. 39, 755–764.

Korzeniewski, C., Callewaert, D.M., 1983. An enzyme-release assay for natural cytotoxicity. J. Immunol. Methods 64, 313–320.

Kroemer, G., Dallaporta, B., Resche-Rigon, M., 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annu. Rev. Physiol. 60, 619–642.

Krumschnabel, G., Ebner, H.L., Hess, M.W., Villunger, A., 2010. Apoptosis and necroptosis are induced in rainbow trout cell lines exposed to cadmium. Aquat. Toxicol. 99, 73–85.

Krumschnabel, G., Manzl, C., Berger, C., Hofer, B., 2005. Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. Toxicol. Appl. Pharmacol. 209, 62–73.

Ku, C.C., Teng, Y.C., Wang, C.S., Lu, C., 2009. Establishment and characterization of three cell lines derived from the rockfish grouper *Epinephelus quoyanus*: use for transgenic studies and cytotoxicity testing. Aquaculture 294, 147–151.

Kumar, V., Abbas, A.K., Fausto, N., Aster, J.C., 2009. Robbins and cotran pathologic basis of disease. Elsevier Health Sciences, Philadelphia.

Kwong, R.W.M., Andres, J.A., Niyogi, S., 2010. Molecular evidence and physiological characterization of iron absorption in isolated enterocytes of rainbow trout (*Oncorhynchus mykiss*): implications for dietary cadmium and lead absorption. Aquat. Toxicol. 99, 343–350.

Lagadic, L., Caquet, T., 1998. Invertebrates in testing of environmental chemicals: are they alternatives?. Environ. Health Perspect. 106, 593–611.

Lage, C.R., Nayak, A., Kim, C.H., 2006. Arsenic ecotoxicology and innate immunity. Int. Comp. Biol. 46, 1040–1054.

Lai, Y.S., John, J.A.C., Lin, C.H., Guo, I.C., Chen, S.C., Fang, K., Chang, C.Y., 2003. Establishment of cell lines from a tropical grouper, *Epinephelus awoara* (Temminck & Schlegel), and their susceptibility to grouper irido- and nodaviruses. J. Fish Dis. 26, 31–42.

Lai, Y.S., Murali, S., Chiu, H.C., Ju, H.Y., Lin, Y.S., Chen, S.C., Guo, I.C., Fang, K., Chang, C.Y., 2001. Propagation of yellow grouper nervous necrosis virus (YGNNV) in a new nodavirus-susceptible cell line from yellow grouper, *Epinephelus awoara* (Temminck & Schlegel), brain tissue. J. Fish Dis. 24, 299–309.

Laing, K., Zou, J., Wang, T., Bols, N., Hirono, I., Aoki, T., Secombes, C.J., 2002. Identification and analysis of an interleukin 8-like molecule in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 26, 433–444.

Laing, K.J., Pilstrom, L., Cunningham, C., Secombes, C.J., 1999. TGF-β3 exists in bony fish. Vet. Immunol. Immunopathol. 72, 45–53.

Lakra, W.S., Swaminathan, T.R., Joy, K.P., 2011. Development, characterization, conservation and storage of fish cell lines: a review. Fish Physiol. Biochem. 37, 1–20.

Lang, E., Lang, F., 2015. Triggers, inhibitors, mechanisms, and significance of eryptosis: the suicidal erythrocyte death. Biomed. Res. Int. 2015, 1–16.

Lang, F., Abed, M., Lang, E., Föller, M., 2014. Oxidative stress and suicidal erythrocyte death. Antioxid. Redox Signal. 862, 1–61.

Lang, F., Lang, E., Föller, M., 2012. Physiology and pathophysiology of eryptosis. Transfus. Med. Hemother. 39, 308–314.

Lang, K.S., Duranton, C., Poehlmann, H., Myssina, S., Bauer, C., Lang, F., Wieder, T., Huber, S.M., 2003. Cation channels trigger apoptotic death of erythrocytes. Cell Death Differ. 10, 249–256.

Lang, K.S., Lang, P.A., Bauer, C., Duranton, C., Wieder, T., Huber, S.M., Lang, F., 2006. Mechanisms of suicidal erythrocyte death. Cell. Physiol. Biochem. 8, 1183–1192.

Lang, U.E., Puls, I., Müller, D.J., Strutz, N., Gallinat, J., 2005. Mechanisms of suicidal erythrocyte death. Cell. Physiol. Biochem. 15, 195–202.

Lazo, J.S., Kondo, Y., Dellapiazza, D., Michalska, A.E., Choo, K.H., Pitt, B.R., 1995. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J. Biol. Chem. 270, 5506–5510.

Lee, J.H., Youm, J.H., Kwon, K.S., 2006a. Mercuric chloride induces apoptosis in MDCK cells. Prov. Med. Pub. Health 39, 199–204.

Lee, T.H., Kim, S.U., Yu, S.L., Kim, S.H., Park, D.S., Moon, H.B., Dho, S.H., Kwon, K.S., Kwon, H.J., Han, Y.H., Jeong, S., Kang, S.W., Shin, H.S., Lee, K.K., Rhee, S.G., Yu, D.Y., 2003. Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. Blood 101, 5033–5038.

Lee, W.K., Abouhamed, M., Thevenod, F., 2006b. Caspase-dependent and -independent pathways for cadmium-induced apoptosis in cultured kidney proximal tubule cells. Am. J. Physiol. Renal Physiol. 291, 823–832.

Li, H., Zhang, S., 2002. *In vitro* cytotoxicity of the organophosphorous insectice methylparathion to FG-9307, the gill cell line of flounder (*Paralichthys olivaceus*). Cell Biol. Toxicol. 18, 235–241.

Li, J.J., Tang, Q., Li, Y., Hu, B.R., Ming, Z.Y., Fu, Q., Qian, J.Q., Xiang, J.Z., 2006. Role of oxidative stress in the apoptosis of hepatocellular carcinoma induced by combination of arsenic trioxide and ascorbic acid. Acta Pharmacol. Sin. 27, 1078–1084.

Li, M., Xia, T., Jiang, C.S., Li, L.J., Fu, J.L., Zhou, Z.C., 2003. Cadmium directly induced the opening of membrane permeability pore of mitochondria which possibly involved in cadmium-triggered apoptosis. Toxicology 194, 19–33.

Li, M., Zheng, Y., Liang, H., Zou, L., Sun, J., Zhang, Y., Qin, F., Liu, S., Wang, Z., 2013. Molecular cloning and characterization of cat, gpx1 and Cu/Zn-sod genes in pengze crucian carp *(Carassius auratus* var. *Pengze*) and antioxidant enzyme modulation induced by hexavalent chromium in juveniles. Comp. Biochem. Physiol. C 157, 310–321.

Li, R.W., Waldbieser, G.C., 2006. Genomic organisation and expression of the natural killer cell enhancing factor (NKEF) gene in channel catfish, *Ictalurus punctatus* (Rafinesque). Fish Shellfish Immunol. 20, 72–82.

Liu, S., Li, Q., Liu, Z., 2013. Genome-wide identification, characterization and phylogenetic analysis of 50 catfish ATP-binding cassette (ABC) transporter genes. PLoS One 8, e63895.

Liu, Z., 2010. Roles of vertebrate aquaglyceroporins in arsenic transport and detoxification. Adv. Exp. Med. Biol. 679, 71–81.

Liu, Z., Sánchez, M.A., Jiang, X., Boles, E., Landfear, S.M., Rosen, B.P., 2006. Mammalian glucose permease GLUT1 facilitates transport of arsenic trioxide and methylarsonous acid. Biochem. Biophys. Res. Commun. 351, 424–430.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.

Livingstone, D.R., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Rev. Med. Vet. 154, 427–430.

Livingstone, D.R., 1993. Review biotechnology and pollution monitoring : use of molecular biomarkers in the aquatic environment. J. Chem. Tech. Biotechnol. 57, 195–211.

Lock, R.A.C., van Overbeeke, A.P., 1981. Effects of mercuric chloride and methylmercuric chloride on mucus secretion in rainbow trout, *Salmo Gairdneri* Richardson. Comp. Biochem. Physiol. C 69, 67–73.

Long, Y., Li, Q., Cui, Z., 2011a. Molecular analysis and heavy metal detoxification of ABCC1/MRP1 in zebrafish. Mol.Biol. Rep. 38, 1703–1711.

Long, Y., Li, Q., Li, J., Cui, Z., 2011b. Molecular analysis, developmental function and heavy metal-induced expression of ABCC5 in zebrafish. Comp. Biochem. Physiol. B. 158, 46–55.

Loo, G.H., Sutton, D.L., Schuller, K.A., 2012. Cloning and functional characterisation of a peroxiredoxin 1 (NKEF A) cDNA from Atlantic salmon (*Salmo salar*) and its expression in fish infected with *Neoparamoeba perurans*. Fish Shellfish Immunol. 32, 1074–1082.

López, E., Figueroa, S., Oset-Gasque, M.J., González, M.P., 2003. Apoptosis and necrosis: two distinct events induced by cadmium in cortical neurons in culture. Brit. J. Pharmacol. 138, 901–911.

Loro, V.L., Jorge, M.B., Silva, K.R. Wood, C.M., 2012. Oxidative stress parameters and antioxidant response to sublethal waterborne zinc in a euryhaline teleost *Fundulus heteroclitus*: Protective effects of salinity. Aquat. Toxicol. 110–111, 187–193.

Low, F.M., Hampton, M.B., Winterbourn, C.C., 2008. Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. Antioxid. Redox Signal 10, 1621–1630.

Low, K.W., Sin, Y.M., 1998. Effects of mercuric chloride and sodium selenite on some immune responses of blue gourami, *Trichogaster trichopterus* (Pallus). Sci. Total Environ. 214, 153–164.

Lu, D.Q., Bei, J.X., Feng, L.N., Zhang, Y., Liu, X.C., Wang, L., Chen, J.L., Lin, H.R., 2008. Interleukin-1beta gene in orange-spotted grouper, *Epinephelus coioides*: molecular cloning, expression, biological activities and signal transduction. Mol. Immunol. 45, 857–867.

Lu, J., Jin, T., Nordberg, G., Nordberg, M., 2001. Metallothionein gene expression in peripheral lymphocytes from cadmium-exposed workers. Cell Stress Chaperon. 6, 97–104.

Lu, Y., Bhushan, S., Tchatalbachev, S., Marconi, M., Bergmann, M., Weidner, W., Chakraborty, T., Meinhardt, A., 2013. Necrosis is the dominant cell death pathway in uropathogenic *Escherichia coli* elicited epididymo-orchitis and is responsible for damage of rat testis. PLoS One 8, e52919.

Lukiw, W.J., McLachlan, D.R., 1995. Aluminum neurotoxicity. In: Chang, L.W., Dyer, R.S. (Eds). Handbook of Neurotoxicology. Marcel-Dekker, New York, pp. 105–142.

Lund, S., Lund, M., Tufts, B., 2003. Red blood cell Hsp70 mRNA and protein as bioindicators of temperature stress in the brook trout (*Salvelinus fontinalis*). Can. J. Fish Aquat. Sci. 60, 460–470.

Luque, D., Gómez-Blanco, J., Garriga, D., Brilot, A.F., González, J.M., Havens, W.M., Carrascosa, J.L., Trus, B.L., Verdague, N., Ghabrial, S.A., Castón, J.R., 2014. Cryo-EM near-atomic structure of a dsRNA fungal virus shows ancient structural motifs preserved in the dsRNA viral lineage. Proc. Natl. Acad. Sci. USA. 111, 7641–7646.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.

Luzio, A., Monteiro, S.M., Fontainhas-Fernandes, A.A., Pinto-Carnide, O., Matos, M., Coimbra, A.M., 2013. Copper induced upregulation of apoptosis related genes in zebrafish (*Danio rerio*) gill. Aquat. Toxicol. 128–129, 183–189.

MacDougal, K.C., Johnson, M.D, Burnett, K.G., 1996. Exposure to mercury alters early activation events in fish leukocytes. Environ. Health Perspect. 104, 1102–1106.

Mahmud, H., Föller, M., Lang, F., 2009. Arsenic-induced suicidal erythrocyte death. Arch. Toxicol. 83, 107–113.

Maier, V.H., Dorn, K.V., Gudmundsdottir, B.K., Gudmundsson, G.H., 2008. Characterisation of cathelicidin gene family members in divergent fish species. Mol. Immunol. 45, 3723–3730.

Maracine, M., Segner, H., 1998. Cytotoxicity of metals in isolated fish cells: importance of the cellular glutathione status. Comp. Biochem. Physiol. A 120, 83–88.

Marnett, L.J., 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361–370.

Martínez-López, E., María-Mojica, P., Martínez, J.E., Calvo, J.F., Romero, D., García-Fernández, A.J., 2005. Cadmium in feathers of adults and blood of nestlings of

three raptor species from a nonpolluted Mediterranean forest, southeastern Spain. Bull. Environ. Contam. Toxicol. 74, 477–484.

Masters, B.A., Quaife, C.J., Erickson, J.C., Kelly, E.J., Froelick, G.J., Zambrowicz, B.P., Brinster, R.L., Palmiter, R.D., 1994. Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles. J. Neurosci. 14, 5844–5857.

Matés J.M., Segura, J.A., Alonso, F.J. Márquez, J., 2010. Roles of dioxins and heavy metals in cancer and neurological diseases using ROS-mediated mechanisms. Free Radic. Biol. Med. 49, 1328–1341.

Matsui, M., Nishigori, C., Toyokuni, S., Takada, J., Akaboshi, M., Ishikawa, M., Imamura, S., Miyachi, Y., 1999. The role of oxidative DNA damage in human arsenic carcinogenesis: detection of 8-hydroxy-2'-deoxyguanosine in arsenic-related Bowen's disease. J. Invest. Dermatol. 113, 26–31.

Mayer, M.P., Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol. Life Sci. 62, 670–684.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: and enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049−6055.

McDermott, J.R., Jiang, X., Beene, L.C., Rosen, B.P., Liu, Z., 2010. Pentavalent methylated arsenicals are substrates of human AQP9. BioMetals 23, 119–127.

McGee, H.M., Woods, G.M., Bennett, B., Chung, R.S., 2010. The two faces of metallothionein in carcinogenesis: photoprotection against UVR-induced cancer and promotion of tumour survival. Photochem. Photobiol. Sci. 9, 586–596.

Mela, M., Neto, F.F., Yamamoto, F.Y., Almeida, R., Grotzner, S.R., Ventura, D.F., Oliveira-Ribeiro, C.A., 2014. Mercury distribution in target organs and biochemical responses after subchronic and trophic exposure to neotropical fish *Hoplias malabaricus*. Fish Physiol. Biochem. 40, 245–256.

Mendil, D., Ünal, Ö.F., Tüzen, M., Soylak, M., 2010. Determination of trace metals in different fish species and sediments from the River Yesilirmak in Tokat, Turkey. Food Chem.Toxicol. 48, 1383–1392.

Meyer, J.N., Smith, J.D., Winston, G.W., Di Giulio, R.T., 2003. Antioxidant defenses in killifish (*Fundulus heteroclitus*) exposed to contaminated sediments and model prooxidants: short-term and heritable responses. Aquat. Toxicol. 65, 377–395.

Mieiro, C.L., Ahmad, I., Pereira, M.E., Duarte, A.C., Pacheco, M., 2010. Antioxidant system breakdown in brain of feral golden grey mullet (*Liza aurata*) as an effect of mercury exposure. Ecotoxicology 19, 1034–1045.

Mieiro, C.L., Bervoets, L., Joosen, R., Blust, R., Duarte, A.C., Pereira, M.E., Pacheco, M., 2011. Metallothioneins failed to reflect mercury external levels of exposure and bioaccumulation in marine fish. Considerations on tissue and species specific responses. Chemosphere 85, 114–121.

Minganti, V., Drava, G., de Pellegrini, R., Siccardi, C., 2010. Trace elements in farmed and wild gilthead seabream, *Sparus aurata*. Mar. Pollut. Bull. 60, 2022–2025.

Minghetti, M., Leaver, M.J., Taggart, J.B., Casadei, E., Auslander, M., Tom, M., George, S.G., 2011. Copper induces Cu-ATPase ATP7A mRNA in a fish cell line, SAF1. Comp. Biochem. Physiol. C 154, 93–99.

Minghetti, M., Schnell, S., Chadwick, M.A., Hogstrand, C., Bury, N.R., 2014. A primary fish gill cell system (FIGCS) for environmental monitoring of river waters. Aquat. Toxicol. 154, 184–192.

Mitchelmore, C.L., Chipman, J.K., 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat. Res. 399, 135–147.

Moffatt, P., Denizeau, F., 1997. Metallothionein in physiological and physiopathological processes. Drug Metab. Rev. 29, 261–307.

Monnet-Tschudi, F., Zurich, M.G., Honegger, Paul., 1996. Comparison of the developmental effects of two mercury compounds on glial cells. Brain Res. 741, 52–59.

Monteiro, D.A., Rantin, F.T., Kalinin, A.L., 2010. Inorganic mercury exposure: toxicological effects, oxidative stress biomarkers and bioaccumulation in the

tropical freshwater fish matrinxã, *Brycon amazonicus* (Spix and Agassiz, 1829). Ecotoxicology 19, 105–123.

Monteiro, D.A., Rantin, F.T., Kalinin, A.L., 2013. Dietary intake of inorganic mercury: bioaccumulation and oxidative stress parameters in the neotropical fish *Hoplias malabaricus*. Ecotoxicology 22, 446–456.

Monteiro, V., Cavalcante, D., Vilela, M., Sofia, S., Martinez, C., 2011. *In vivo* and *in vitro* exposures for the evaluation of the genotoxic effects of lead on the Neotropical freshwater fish *Prochilodus lineatus*. Aquat. Toxicol. 104, 291–298.

Morcillo, P., Cordero, H., Meseguer, J., Esteban, M.A., Cuesta, A., 2015a. Toxicological *in vitro* effects of heavy metals on gilthead seabream (*Sparus aurata* L.) head-kidney leucocytes. Toxicol. In Vitro 30, 412–420.

Morcillo, P., Cordero, H., Meseguer, J., Esteban, M.A., Cuesta, A., 2015b. *In vitro* immunotoxicological effects of heavy metals on European sea bass (*Dicentrarchus labrax* L.) head-kidney leucocytes. Fish Shellfish Immunol. 47, 245–254.

Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225–233.

Mori, M., Wakabayashi, M., 2000. Cytotoxicity evaluation of chemicals using cultured fish cells. Water Sci. Technol. 42, 277–282.

Morimoto, R.I., 2011. The heat shock response: systems biology of proteotoxic stress in aging and disease. Cold Spring Harb. Symp.76, 91–99.

Mosmann, 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mourich, D.V., Hansen, J., Leong, J., 1995. Natural killer cell enhancement factor-like gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics 42, 438–439.

Moyes, C.D., Sharma, M.L., Lyons, C., Leary, S.C., Leon, M., Petrie, A., Lund, S.G., Tufts, B.L., 2002. Origins and consequences of mitochondrial decline in nucleated erythrocytes. Biochim. Biophys. Acta 1591, 11–20.

Mulero, V., Esteban, M.A, Meseguer, J., 1998. Effects of *in vitro* addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes. Vet. Immunol. Immunopathol. 66, 185–199.

Mykkanen, H.M., Ganther, H.E., 1974. Effect of mercury on erythrocyte glutathione reductase activity. *In vivo* and *in vitro* studies. Bull. Environ. Contam. Toxicol. 12, 10–16.

Nagai, T., Yukimoto, T., Suzuki, N., 2002. Glutathione peroxidase from the liver of Japanese sea bass *Lateolabrax japonicus*. Z. Naturforsch C 57, 172–176.

Naicker, D., Myburgh, J.G., Botha, C.J., 2007. Establishment and validation of primary hepatocytes of the African sharptooth catfish (*Clarias gariepinus*). Chemosphere 68, 69–77.

Navarro, A., Quirós, L., Casado, M., Faria, M., Carrasco, L., Benejam, L., Benito, J., Díez, S., Raldúa, D., Barata, C., Bayona, J.M., Piña, B., 2009. Physiological responses to mercury in feral carp populations inhabiting the low Ebro River (NE Spain), a historically contaminated site. Aquat. Toxicol. 93, 150–157.

Nayak, A.S., Lage, C.R., Kim, C.H., 2007. Effects of low concentrations of arsenic on the innate immune system of the zebrafish (*Danio rerio*). Toxicol. Sci. 98, 118–124.

Neumann, C.A., Krause, D.S., Carman, C.V., Das, S., Dubey, D.P., Abraham, J.L., Bronson, R.T., Fujiwara, Y., Orkin, S.H., Van Etten, R.A., 2003. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. Nature 424, 561–565.

Niemi, G.J., McDonald, M.E., 2004. Application of ecological indicators. Annu. Rev. Ecol. Evol. Syst. 35, 89–111.

Niki, E., Yoshida, Y., Saito, Y., Noguchi, N., 2005. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem. Biophys. Res. Commun. 338, 668–676.

Nishida, Y., 2011. The chemical mechanism of oxidative stress by copper(II) and iron(III) ions in several neurodegenerative disorders. Metal ions in Neurological Systems. Springer-Verlag ,Wien, pp. 163–172.

Niyogi, S., Kent, R., Wood, C.M., 2008. Effects of water chemistry variables on gill binding and acute toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*): a biotic ligand model (BLM) approach. Comp. Biochem. Physiol. C 148, 305–314.

Niyogi, S., Wood, C.M., 2004. Critical review biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.

Norbury, C.J., Hickson, I.D., 2001. Cellular responses to DNA damage. Annu. Rev. Pharmacol. Toxicol. 41, 367–401.

Ofengeim, D., Yuan, J., 2013. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nature, 14, 727–736.

O'Neill, J.G., 1981. The humoral response of *Salmo trutta* L. and *Cyprinus carpio* L. exposed to heavy metals. J. Fish Biol. 19, 297–306.

Oliveira-Ribeiro, C.A., Belger, L., Pelletier, É., Rouleau, C., 2002. Histopathological evidence of inorganic mercury and methyl mercury toxicity in the arctic charr (*Salvelinus alpinus*). Environ. Res. 90, 217–225.

Oliveira-Ribeiro, C.A., Fanta, E., Turcatti, N.M., Cardoso, R.J., Carvalho, C.S., 1996. Lethal effects of inorganic mercury on cells and tissues of *Trichomycterus brasiliensis* (Pisces; Siluroidei). Biocell 20, 171–178.

Olsen, R.E., Lovaas, E., Lie, O., 1999. The influence of temperature, dietary polyunsaturated fatty acids, alpha-tocopherol and spermine on fatty acid composition and indices of oxidative stress in juvenile Atlantic char, *Salvelinus aplinus* (L.). Fish Physiol. Biochem. 20, 13–29.

Orbea, A., Beier, K., Völkl, A., Fahimi, H.D., Cajaraville, M.P., 1999. Ultrastructural, immunocytochemical and morphometric characterization of liver peroxisomes in gray mullet, *Mugil cephalus*. Cell Tissue Res. 297, 493–502.

Ormerod, M.G., 1990. Analysis of DNA. General methods. In: Ormerod, M.G. (Ed). Flow cytometry: a practical approach. Oxford University, Oxford, pp. 69–87.

Paetzold, S.C., Ross, N.W., Richards, R.C., Jones, M., Hellou, J., Bard, S.M., 2009. Up-regulation of hepatic ABCC2, ABCG2, CYP1A1 and GST in multixenobiotic-resistant killifish (*Fundulus heteroclitus*) from the Sydney Tar Ponds, Nova Scotia, Canada. Mar. Environ. Res. 68, 37–47.

Palmieri, M.A., Tasat, D.R., Molinari, B.L., 2007. Oxidative metabolism of lung macrophages exposed to sodium arsenite. Toxicol. In Vitro 21, 1603–1609.

Parihar, M.S., Dubey, A.K., 1995. Lipid peroxidation and ascorbic acid status in respiratory organs of male and female freshwater catfish *Heteropneustes fossilis* exposed to temperature increases. Comp. Biochem. Physiol. C 112, 309–313.

Park, J.D., Liu, Y., Klaassen, C.D., 2001. Protective effect of metallothionein against the toxicity of cadmium and other metals. Toxicology 163, 93–100.

Patrick, L., 2003. Toxic metals and antioxidants. Part II. The role of antioxidants in arsenic and cadmium toxicity. Altern. Med. Rev. 8, 106–128.

Paul, I., Mandal, C., Mandal, C., 1998. Effect of environmental pollutants on the C-reactive protein of a freshwater major carp, *Catla catla*. Dev. Comp. Immunol. 22, 519–532.

Payne, J.F., Malins, D.C., Gunselman, S., Rahimtula, A., Yeats, P.A., 1998. DNA oxidative damage and vitamin A reduction in fish from a large lake system in Labrador, Newfoundland, contaminated with iron ore mine tailings. Mar. Environ. Res. 46, 289–294.

Pedlar, R.M., Ptashynski, M.D., Wautier, K.G., Evans, R.E., Baron, C.L., Klaverkamp, J.F., 2002. 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, 73–91.

Penny, C., Adams, C., 1863. Fourth report, royal commission on pollution of rivers in Scotland. Evidence, London, pp. 377–391.

Perea, G., Sur, M., Araque, A., 2014. Neuron-glia networks: integral gear of brain function. Front. Cell. Neurosci. 8,1–8.

Pérez-Sánchez, J., Bermejo-Nogales, A., Calduch-Giner, J.A., Kaushik, S., Sitjà-Bobadilla, A., 2011. Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream (*Sparus aurata*): insights from fish exposed to dietary, pathogen and confinement stressors. Fish Shellfish Immunol. 31, 294–302.

Ponce, R.A., Kavanagh, T.J., Mottet, N.K., Whittaker, S.G., Faustman, E.M., 1994. Effects of methyl mercury on the cell cycle of primary rat CNS cells *in vitro*. Toxicol. Appl. Pharm. 127, 83–90.

Press, C.M., Dannevig, B.H., Landsverk, T., 1994. Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. 4, 79–93.

Pressley, M.E., Phelan, P.E., Eckhard, W.P., Mellon, M.T., Kim, C.H., 2005. Pathogenesis and inflammatory response to *Edwardsiella tarda* infection in the zebrafish. Dev. Comp. Immunol. 29, 501–513.

Pulido, M.D., Parrish, A.R., 1993. Metal-induced apoptosis: mechanisms. Mut. Res. 533, 227–241.

Rachlin, J.W., Perlmutter, A., 1968. Fish cells in culture for study of aquatic toxicants. Water Res. 2, 409–414.

Rajeshkumar, S., Mini, J., Munuswamy, N., 2013. Effects of heavy metals on antioxidants and expression of HSP70 in different tissues of Milk fish (*Chanos chanos*) of Kaattuppalli Island, Chennai, India. Ecotox. Environ. Safe. 98, 8–18.

Rakers, S., Klinger, M., Kruse, C., Gebert, M., 2011. Pros and cons of fish skin cells in culture: long-term full skin and short-term scale cell culture from rainbow trout, *Oncorhynchus mykiss*. Eur. J. Cell Biol. 90, 1041–1051.

Rana, S.V., 2008. Metals and apoptosis: recent developments. J. Trace Elem. Med. Biol. 22, 262–284.

Rana, S.V.S., Singh, R., Verma, S., 1995. Mercury-induced lipid peroxidation in the liver, kidney, brain and gills of a fresh water fish *Channa punctatus*. Jpn. J. Ichthyol. 42, 255–259.

Randall, D.J., Perry, S.F., 1992. Catecholamine. In: Hoar, W.S., Randall, D.J. Farrell, T. P. (Eds). Fish physiology. Academic Press, New York. pp. 255–300.

Reinhart, B., Lee, L., 2002. Integrin-like substrate adhesion in RTG-2 cells, a fibroblastic cell line derived from rainbow trout. Cell Tissue Res. 307, 165–172.

Risso-De Faverney, C., Devaux, A., Lafaurie, M., Girard, J.P., Bailly, B., Rahmani, R., 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species. Aquat. Toxicol. 53, 65–76.

Risso-De Faverney, C., Orsini, N., De Sousa, G., Rahmani, R., 2004. Cadmium-induced apoptosis through the mitochondrial pathway in rainbow trout hepatocytes: involvement of oxidative stress. Aquat. Toxicol. 69, 247–258.

Roberts, R.J., Agius, C., Saliba, C., Bossier, P., Sung, Y.Y., 2010. Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. J. Fish Dis. 33, 789–801.

Rocco, L., Valentino, I.V., Scapigliati, G., Stingo, V., 2014. RAPD-PCR analysis for molecular characterization and genotoxic studies of a new marine fish cell line derived from *Dicentrarchus labrax*. Cytotechnology 66, 383–393.

Roche, H., Bogé, G., 1993. Effects of Cu, Zn and Cr salts on antioxidant enzyme activities *in vitro* of red blood cells. Toxicol. In Vitro 7, 623–629.

Roche, H., Bogé, G., 2000. *In vivo* effects of phenolic compounds on blood parameters of a marine fish (*Dicentrarchus labrax*). Comp. Biochem. Phys. C 125, 345–353.

Rodríguez, A., Esteban, M.A., Meseguer, J., 2003. Phagocytosis and peroxidase release by seabream (*Sparus aurata* L.) leucocytes in response to yeast cells. Anat. Rec. A Discov. Mol. Cell. Evol. Biol. 272, 415–423.

Rogers, J.T.,Wood, C.M., 2004. Characterization of branchial lead-calcium interaction in the freshwater rainbow trout *Oncorhynchus mykiss*. J. Exp. Biol. 207, 813–825.

Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., Girard, J.P., 2000. Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. Aquat. Toxicol. 48, 185–194.

Romero, D., Gómez-Zapata, M., Luna, A., García-Fernández, A.J., 2003. Morphological characterisation of BGM (*Buffalo Green Monkey*) cell line exposed to low doses of cadmium chloride. Toxicol. In Vitro 17, 293–299.

Romero, D., Gómez-Zapata, M., Luna, A; García-Fernández, A.J., 2000. Comparación de dos métodos colorimétricos en la evaluación citotóxica de metales pesados en cultivos de células renales. Rev. Toxicol. 17, 91–95.

Rotchell, J.M., Clarke, K.R., Newton, L.C., Bird, D.J., 2001. Hepatic metallothionein as a biomarker for metal contamination: age effects and seasonal variation in European flounders (*Pleuronectes flesus*) from the Severn Estuary and Bristol Channel. Mar. Environ. Res. 52, 151–171.

Roth, V., 2006. Doubling Time Computing. Available from: http://www.doubling-

time.com/compute.php.

Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., Kizek, R., 2013. The role of metallothionein in oxidative stress. Int. J. Mol. Sci. 14, 6044–6066.

Said, K., Ali, A., Ferencz, J., Nemcsók, J., Hermesz, E., 2010. Expression of heat shock and metallothioneins genes in the heart of common carp (*Cyprinus carpio*): effects of the temperature shock and heavy metal exposure. Acta Biol. Hung. 61, 10–23.

Salinas, I., Rodríguez, A., Meseguer, J., Esteban, M.A., 2007. Adenosine arrests apoptosis in lymphocytes but not in phagocytes from primary leucocyte cultures of the teleost fish, *Sparus aurata* L. Dev. Comp. Immunol. 31, 1233–1241.

Salinas, I., Zhang, Y.A., Sunyer, J.O., 2011. Mucosal immunoglobulins and B cells of teleost fish. Dev. Comp. Immunol. 35, 1346–1365.

Shakunthala, N., 2010. New cytochrome P450 mechanisms: implications for understanding molecular basis for drug toxicity at the level of the cytochrome. Expert. Opin. Drug. Metab. Toxicol. 6, 1–15.

Sánchez-Dardon J., Voccia, I., Hontela, A., Anderson, P., Brousseau, P., Blakely, B., Fournier, M., 1997. Immunotoxicity of cadmium, zinc and mercury after *in vivo* exposure, alone or in mixture in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 21, 129–133.

Sánchez-Dardon, J., Voccia, I., Hontela, A, Chilmonczyk, S., Dunier, M., Boermans, H., Blakley, B., Fournier, M., 1999. Immunomodulation by heavy metals tested individually or in mixtures in rainbow trout (*Oncorhynchus mykiss*) exposed *in vivo*. Environ. Toxicol. Chem. 18, 1492–1497.

Sancho, P., Fernandez, C., Yuste, V.J., Amran, D., Ramos, A.M., Blas, E., Susin, S.A., Aller, P., 2006. Regulation of apoptosis/necrosis execution in cadmium-treated

human promonocytic cells under different forms of oxidative stress. Apoptosis 11, 673–686.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an enviromental perspective. Crit. Rev. Toxicol. 23, 49–75.

Sandrini, J.Z., Bianchini, A., Trindade, G.S., Nery, L.E.M., Marins, L.F.F., 2009. Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish *(Danio rerio*) ZFL cells. Aquat. Toxicol. 95, 285–291.

Sapkota, A., Sapkota, A.R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., Lawrence, R., 2008. Aquaculture practices and potential human health risks: current knowledge and future priorities. Environ. Int. 34, 1215–1226.

Sarmento, A., Guilhermino, L., Afonso, A., 2004. Mercury chloride effects on the function and cellular integrity of sea bass (*Dicentrarchus labrax*) head kidney macrophages. Fish Shellfish Immunol. 17, 489–498.

Sassi, A., Darias, M.J., Said, K., Messaoudi, I., Gisbert, E., 2013. Cadmium exposure affects the expression of genes involved in skeletogenesis and stress response in gilthead sea bream larvae. Fish Physiol. Biochem. 39, 649–659.

Sathiyaa, R., Vijayan, M.M., 2003. Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Am. J. Physiol. Cell Physiol. 284, 1508–1515.

Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquac. Res. 39, 223–239.

Savan, R., Aman, A., Sato, K., Yamaguchi, R., Sakai, M., 2005. Discovery of a new class of immunoglobulin heavy chain from fugu. Eur. J. Immunol. 35, 3320–3331.

Scapigliati, G., Bird, S., Secombes, C.J., 2000. Invertebrate and fish cytokines. Eur. Cytokine Netw. 11, 354–361.

Schirmer, K., 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology 224, 163–183.

Schirmer, K., Chan, A.G., Bols, N.C., 2000. Transitory metabolic disruption and cytotoxicity elicited by benzo[a]pyrene in two cell lines from rainbow trout liver. J. Biochem. Mol. Toxicol. 14, 262–276.

Schirmer, K., Dixon, D.G., Greenberg, B.M., Bols, N.C., 1998. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicology 127, 129–141.

Schlenk, D., Handy, R., Steinert, S., Depledge, M., Benson, W., 2008. "Biomarkers." In: Di Giulio, R.T., Hinton, D.E. (Eds). The toxicology of fishes. CRC Press, Florida, USA, pp. 683–715.

Schull, S., Heintz, N.H., Periasamy, M., Manohar, M., Janssen, Y.M.W., Marsh, J.P., Mossman, B.T., 1991. Differential regulation of antioxidant enzymes in response to oxidants. J. Biol. Chem. 266, 24398–24403.

Schurz, F., Sabater-Vilar, M., Fink-Gremmels, J., 2000. Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins. Mutagenesis 15, 525–530.

Schuwerack, P.M.M., Lewis, J.W., Hoole, D., 2003. Cadmium-induced cellular and immunological responses in *Cyprinus carpio* infected with the blood parasite, *Sanguinicola inermis*. J. Helminthol. 77, 341–350.

Schwarz, M.A., Lazo, J.S., Yalowich, J.C., Allen, W.P., Whitmore, M., Bergonia, H.A., Tzeng, E., Billiar, T.R., Robbins, P.D., Lancaster, J.R., Pitt, B.R., 1995. Metallothionein protects against the cytotoxic and dna-damaging effects of nitric-oxide. Proc. Natl. Acad. Sci. USA. 92, 4452–4456.

Scott, M.D., Eaton, J.W., Kuypers, F.A., Chiu, D.T., Lubin, B.H., 1989. Enhancement of erythrocyte superoxide dismutase activity: effects on cellular oxidant defense. Blood 74, 2542–2549.

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C., Zou, J., 2001. Cytokines and innate immunity of fish. Dev. Comp. Immunol. 25, 713–723.

Segner, H., 1998. Fish cell lines as a tool in aquatic toxicology. In: Braunbeck, T., Hinton, D.E., Streit, B. (Eds). Fish ecotoxicology. Switzerland, Birkhäuser Basel, pp. 1–38.

Segner, H., 2004. Cytotoxicity assays with fish cells as an alternative to the acute lethality test with fish. Atla 32, 375–382.

Segner, H., Lenz, D., 1993. Cytotoxicity assays with the rainbow trout Rl cell line. Toxicol. In Vitro 7, 537–540.

Segner, H., Lenz, D., Hanke, W., Schüürmann, G., 1994. Cytotoxicity of metals towards rainbow trout R1 cell line. Environ. Toxicol. Water 9, 273–279.

Sekar, D., Falcioni, M., 2014. DNA damage and repair following *in vitro* exposure to two different forms of titanium dioxide nanoparticles on trout erythrocyte. Environ. Toxicol. 29, 117–127.

Selvaraj, V., Armistead, M.Y., Cohenford, M., Murray, E., 2013. Arsenic trioxide (As_2O_3) induces apoptosis and necrosis mediated cell death through mitochondrial membrane potential damage and elevated production of reactive oxygen species in PLHC-1 fish cell line. Chemosphere 90, 1201–1209.

Selvaraj, V., Yeager-Armstead, M., Murray, E., 2012. Protective and antioxidant role of selenium on arsenic trioxide-induced oxidative stress and genotoxicity in the fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 31, 2861–2869.

Sengupta, M., Bishayi, B., 2002. Effect of lead and arsenic on murine macrophage response. Drug Chem. Toxicol. 25, 459–472.

Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Ryu, D.Y., Park, J.H., 2007. Arsenite-induced apoptosis is prevented by antioxidants in zebrafish liver cell line. Toxicol. In Vitro 21, 870–877.

Serra-Majem, L., Román-Viñas, B., Salvador, G., Ribas-Barba, L., Ngo, J., Castell, C., Cabezas, C., 2007. Knowledge, opinions and behaviours related to food and nutrition in Catalonia, Spain (1992-2003). Public Health Nutr. 10, 1396–13405.

Servili, A., Bufalino, M.R., Nishikawa, R., Sánchez-Melo, I., Muñoz-Cueto, J.A., Lee, L.E.J., 2009. Establishment of long term cultures of neural stem cells from adult sea bass, *Dicentrarchus labrax*. *Comp*. Biochem. Physiol. A 152, 245–254.

Shah, S.L., Altindag, A., 2004. Haematological parameters of tench (*Tinca tinca* L., 1758) on acute and chronic exposures of lethal and sublethal treatments of cadmium. Fresenius Environ. Bull. 13, 1477–1481.

Shah, S.L., Altindag, A., 2005. Alterations in the immunological parameters of tench (*Tinca tinca* L. 1758) after acute and chronic exposure to lethal and sublethal treatments with mercury cadmium and lead. Turk. J. Anim. Sci. 29, 1163–1168.

Shannon, L., Winski, D.C., 1998. Arsenate toxicity in human erythrocytes: characterization of morphologic changes and determination of the mechanism of damage. J. Toxicol. Environ. Health A 53, 345–355.

Sharifi, A.M., Baniasadi, S., Jorjani, M., Rahimi, F., Bakhshayesh, M., 2002. Investigation of acute lead poisoning on apoptosis in rat hippocampus *in vivo*. Neurosci. Lett. 329, 45–48.

Sharp, G.J.E., Secombes, C.J., 1993. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immunol. 3, 119–129.

Shaw, C.F., Savas, M.M., Petering, D.H., 1991. Ligand substitution and sulfhydryl reactivity of metallothionein. Methods Enzymol. 205, 401–414.

Shih, C.M., Wu, J.S., Ko, W.C., Wang, L.F., Wei, Y.H., Liang, H.F., Chen, Y.C., Chen, C.T., 2003. Mitochondria-mediated caspase-independent apoptosis induced by cadmium in normal human lung cells. J. Cell. Biochem. 89, 335–347.

Shin, J.H., Lim, K.M., Noh, J.Y., Bae, O.N., Chung, S.M., Lee, M.Y., Chung, J.H., 2007. Lead-induced procoagulant activation of erythrocytes through

phosphatidylserine exposure may lead to thrombotic diseases. Chem. Res. Toxicol. 20, 38–43.

Shinkai, Y., Sumi, D., Toyama, T., Kaji, T., Kumagai, Y., 2009. Role of aquaporin 9 in cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. Toxicol. Appl. Pharmacol. 237, 232–236.

Simons, T.J.B., 1993. Lead transport and binding by human erythrocytes *in vitro*. Pflugers Arch. 423, 307–313.

Singh, V.K., Mishra, K.P., Rani, R., Yadav, V.S., Awasthi, S.K., Garg, S.K., 2003. Immunomodulation by lead. Immunol. Res. 28, 151–166.

Skarmeta, A.M., Bandin, I., Santos, Y., Toranzo, A.E., 1995. *In vitro* killing of *Pasteurella piscicida* by fish macrophages. Dis. Aquat. Organ. 23, 51–57.

Slaninová, A., Smutna, M., Modra, H., Svobodova, Z., 2009. A review: oxidative stress in fish induced by pesticides. Neuroendocrinol. Lett. 30, 2–12.

Smedley, P.L., Kinniburgh, D.G., 2002. A review of the source, behaviour and distribution of arsenic in natural waters. Appl. Geochem. 17, 517–568.

Smeets, J.M.W., Wamsteker, J., Roth, B., Everaarts, J., Van den Berg, M., 2002. Cytochrome P4501A induction and testosterone hydroxylation in cultured hepatocytes of four fish species. Chemosphere 46, 163–172.

Smirnov, L.P., Sukhovskaya, I.V., Nemova, N.N., 2005. Effects on environmental factors on low-molecular-weight peptides of fishes: a review. Russ. J. Ecol. 36, 41–47.

Sonali, K., Doke, Shashikant., C, Dhawale., 2015. Alternatives to animal testing: a review. Saudi Pharm. J. 23, 223–229.

Sopjani, M., Föller, M., Lang, F., 2008. Gold stimulates Ca^{2+} entry into and subsequent suicidal death of erythrocytes. Toxicology 244, 271–279.

Souid, G., Souayed, N., Yaktiti, F., Maaroufi, K., 2013. Effect of acute cadmium exposure on metal accumulation and oxidative stress biomarkers of *Sparus aurata*. Ecotox. Environ. Safe. 89, 1–7.

Sövényi, J., Szakolczai, J., 1993. Studies on the toxic and immunosuppressive effects of cadmium on the common carp. Acta Vet. Hung. 41, 415–426.

Steinhagen, D., Helmus, T., Maurer, S., Michael, R.D., Leibold, W., Scharsack, J.P., Skouras, A., Schuberth, H.J., 2004. Effect of hexavalent carcinogenic chromium on carp *Cyprinus carpio* immune cells. Dis. Aquat. Organ. 62, 155–161.

Sumathy, K., Desai, K.V., Kondaiah, P., 1997. Isolation of transforming growth factor-β2 cDNA from a fish, *Cyprinus carpio* by RT-PCR. Gene 191, 103–107.

Sutton, D.L., Loo, G.H., Menz, R.I., Schuller, K.A., 2010. Cloning and functional characterization of a typical 2-Cys peroxiredoxin from southern bluefin tuna (*Thunnus maccoyii*). Comp. Biochem. Physiol. B 156, 97–106.

Sweet, L.I., Zelikoff, J.T., 2001. Toxicology and immunotoxicology of mercury: a comparative review in fish and humans. J. Toxicol. Environ. Health B 4, 161–205.

Szakács, G., Váradi, A., Özvegy-Laczka, C., Sarkadi, B., 2008. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov. Today 13, 379–393.

Tait, S.W.G., Green, D.R., 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell. Biol. 11, 621–632.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., Inoue, M., 1999. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res. 59, 551–557.

Tan, S., Li, H., Jin, Y., Yu, H., 2014. *In vitro* and *in vivo* effects of sublethal cadmium on the expression of MT2 and ABCC2 genes in grass carp (*Ctenopharyngodon idellus*). Ecotox. Environ. Safe. 108, 258–264.

Tariq, J., Jaffar, M., Ashraf, M., Moazzam, M., 1993. Heavy metal concentrations in fish, shrimp, seaweed, sediment, and water from the Arabian sea, Pakistan. Mar. Pollut. Bull. 26, 644–647.

Teles, M., Mackenzie, S., Boltaña, S., Callol, A., Tort, L., 2011. Gene expression and TNF-alpha secretion profile in rainbow trout macrophages following exposures to copper and bacterial lipopolysaccharide. Fish Shellfish Immunol. 30, 340–346.

Templeton, D.M., Cherian, M.G., 1991. Toxicological significance of metallothionein. Methods Enzymol. 205, 11–24.

Thornalley, P.J., Vasak, M., 1985. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta 827, 36–44.

Tiffany-Castiglioni, E., 1993. Cell culture models for lead toxicity in neuronal cell culture models for lead toxicity in neuronal and glial cells. Neurotoxicology 14, 513–536.

Tocher, D.R., Mourente, G., Van Der Eecken, A., Evjemo, J.O., Diaz, E., Bell, J.G., Geurden, I., Lavens, P., Olsen, Y., 2002. Effects of dietary vitamin E on antioxidant defense mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquacult. Nutr. 8, 195–207.

Tort, L., Balasch, J.C., Mackenzie, S., 2003. Fish immune system. A crossroads between innate and adaptive responses. Immunología 22, 277–286.

Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in

teleost fish: a review. Vet. Med. 56, 486–503.

Uysal, K., Köse, E., Bülbül, M., Dönmez, M., Erdogan, Y., Koyun, M., Omeroglu, C., Ozmal, F., 2009. The comparison of heavy metal accumulation ratios of some fish species in Enne Dame Lake (Kütahya/Turkey). Environ. Monit. Assess. 157, 355–362.

Valero, Y., Martínez-Morcillo, F.J., Esteban, M.A., Chaves-Pozo, E., Cuesta, A., 2015a. Fish peroxiredoxins and their role in immunity. Biology 4, 860–880.

Valero, Y., Morcillo, P., Meseguer, J., Esteban, M.A., Chaves-Pozo, E., Cuesta, A., 2015b. Characterization of the interferon pathway in teleost fish and its role in the viral immune response in the gonad upon viral nervous necrosis virus infection. J. Gen. Virol. 96, 2176–2187.

Valko, M., Morris, H., Cronin, M.T., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161–1208.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Vasák, M., Hasler, D.W., 2000. Metallothioneins: new functional and structural insights. Curr. Opin. Chem. Biol. 4, 177–183.

Vazzana, M., Celi, M., Tramati, C., Ferrantelli, V., Arizza, V., Parrinello, N., 2014. *In vitro* effect of cadmium and copper on separated blood leukocytes of *Dicentrarchus labrax*. Ecotox. Environ. Safe. 102, 113–120.

Vega, M.M., Fernández, C., Blázquez, T., Tarazona. J.V., Castaño, A., 1996. Biological and chemical tools in the toxicological risk assessment of Jarama River, Madrid, Spain. Environ. Pollut. 93, 135–139.

Velma, V., Vutukuru, S.S., Tchounwou, P.B., 2010. Ecotoxicology of hexavalent chromium in freshwater fish: a critical review. Rev. Environ. Health 24, 129–

145.

Vian, L., Vincent, J., Maurin, J., Fabre, I., Giroux, J., Cano, J.P., 1995. Comparison of three *in vitro* cytotoxicity assays for estimating surfactant ocular irritation. Toxicol. In Vitro 9, 185–190.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. C 46, 281–300.

Viola, A., Pregnolato, G.A.V., Albergoni, V., 1996. Effect of *in vitro* cadmium exposure on natural killer (NK) cells of catfish *Ictalurus melas.* Fish Sellfish Immunol. 6, 167–172.

Voccia, I., Krzystyniak, K., Dunier, M., Flipo, D., Fournier, M., 1994. *In vitro* mercury-related cytotoxicity and functional impairment of the immune cells of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 29, 37–48.

Voccia, I., Sánchez-Dardon, J., Dunier, M., Anderson, P., Fournier, M., Hontela, A., 1996. *In vivo* effects of cadmium chloride on the immune response and plasma cortisol of rainbow trout (*Oncorhynchus mykiss*). In: Stolen. J.S., Fletcher, T.C., Bayne, C.J., Secombes, C.J., Zelikoff, T.J., Twerdok, K.E., Anderson, D.P. (Eds). Modulators of immune responses, the evolutionary trail. SOS Publications, Fair Haven, New Jersey, pp. 547–555.

Waisberg, M., Joseph, P., Hale, B., Beyersmann, D., 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology 192, 95–117.

Walter, S., Buchner, J., 2002. Molecular chaperones-celular machines for protein folding. Angew. Chem. Int. Ed. Engl. 2, 1098–1113.

Walsh, M., Lutz, R.J., Cotter, T.G., O'Connor, R., 2002. Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-XL. Blood 99, 3439–3448.

Wang, L., Meece, K., Williams, D.J., Lo, K.A., Zimmer, M., Heinrich, G., Carli, J.M., Leduc, C.A., Sun, L., Zeltser, L.M., Freeby, M., Goland, R., Tsang, S.H., Wardlaw, S, Egli, D., Leibel, R., 2015. Differentiation of hypothalamic-like neurons from human pluripotent stem cells. J. Clin. Invest. 1, 1–13.

Wang, T.S., Kuo, C.F., Jan, K.Y., Huang, H.M., 1996. Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. J. Cell. Physiol. 169, 256–268.

Wang, X.L., Wang, N., Sha, Z.X., Chen, S.L., 2010. Establishment, characterization of a new cell line from heart of half smooth tongue sole (*Cynoglossus semilaevis*). Fish Physiol. Biochem. 36, 1181–1189.

Wang, Y., Fang, J., Leonard, S.S., Rao, K.M., 2004a Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic Biol. Med. 36, 1434–1443.

Wang, Y.C., Chaung, R.H., Tung, L.C., 2004b. Comparison of the cytotoxicity induced by different exposure to sodium arsenite in two fish cell lines. Aquat. Toxicol. 69, 67–79.

Wang, Z.F., Guo, X., 2011. Arsenite-induced apoptosis is prevented by selenite in A375 cell line. Biol. Trace Elem. Res. 140, 7–17.

Wen, C.M., Cheng, Y.H., Huang, Y.F., Wang, C.S., 2008a. Isolation and characterization of a neural progenitor cell line from tilapia brain. Comp. Biochem. Physiol. A 149, 167–180.

Wen, C.M., Lee, C.W., Wang, C.S., Cheng, Y.H., Huang, H.Y., 2008b. Development of two cell lines from *Epinephelus coioides* brain tissue for characterization of betanodavirus and megalocytivirus infectivity and propagation. Aquaculture 278, 14–21.

Wen, C.M., Wang, C.S., Chin, T.C., Cheng, S.T., Nan, F.H., 2010. Immunochemical and molecular characterization of a novel cell line derived from the brain of *Trachinotus blochii* (Teleostei, Perciformes): a fish cell line with

oligodendrocyte progenitor cell and tanycyte characteristics. Comp. Biochem. Physiol. A 156, 224–231.

Wester, P.W., Vethaak, A.D., van Muiswinkel, W.B., 1994. Fish as biomarkers in immunotoxicology. Toxicology 86, 213–232.

Winzer, K., Becker, W., Van Noorden, C.J.F., Köhler, A., 2000. Short-time induction of oxidative stress in hepatocytes of the European flounder (*Platichthys flesus*). Mar. Environ. Res. 50, 495–501.

Witeska, M., 2004. The effect of toxic chemicals on blood cell morphology in fish. Fresen. Environ. Bull. 13, 1379–1384.

Witeska, M., 2013. Erythrocytes in teleost fishes: a review. Zool. Ecol. 23, 275–281.

Witeska, M., Kondera, E., Szczygielska, K., 2011. The effects of cadmium on common carp erythrocyte morphology. Polish J. Environ. Stud. 20, 783–788.

Witeska, M., Wakulska, M., 2007. The effects of heavy metals on common carp white blood cells *in vitro*. Altern. Lab. Anim. 35, 87–92.

Wolf, K., 1988. Fish viruses and fish viral diseases. In: Wolf, K. (Ed). Cornell University Press, Ithaca, New York, pp. 217–249.

Wolf, K., Quimby, C., 1976. Primary monolayer culture of fish cells initiated from trypsinized tissues. TCA Manual 2, 453–456.

Woods, J.S., Dieguez–Acuna, F.J., Ellis, M.E., Kushleika, J., Simmonds, P.L., 2002. Attenuation of nuclear factor kappa B (NF-κB) promotes apoptosis of kidney epithelial cells: a potential mechanism of mercury–induced nephrotoxicity. Environ. Health Perspect. 110, 819–822.

Wright, R.O., Baccarelli, A., 2011. Metals and neurotoxicology. J. Nutr. 137, 2809–2813.

Wu, S.M., Shih, M.J., Ho, Y.C., 2007. Toxicological stress response and cadmium distribution in hybrid tilapia (*Oreochromis* sp.) upon cadmium exposure. Comp. Biochem. Physiol. C 145, 218–226.

Xiang, L.X., Shao, J.Z., Meng, Z., 2001. Apoptosis induction in fish cells under stress of six heavy metal ions. Prog. Biochem. Biophys. 28, 866–886.

Xu, Z., Parra, D., Gómez, D., Salinas, I., Zhang, Y.A., Jørgensen, L.V.G., Heinecke, R.D., Buchmann, K., La Patra, S., Sunyer, J.O., 2013. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. Proc. Natl. Acad. Sci. USA. 110, 13097–13102.

Xu, Z., Takizawa, F., Parra, D., Gómez, D, Jørgensen, L.V.G, La Patra, S, Sunyer, J.O., 2016. Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods. Nat Commun. 12, 7–10728.

Yang, H., Rose, N.L., 2003. Distribution of mercury in six lake sediment cores across the UK. Sci. Total. Environ. 304, 391–404.

Yang, P.M., Chen, H.C., Tsai, J.S., Lin, L.Y., 2007. Cadmium induces Ca^{2+}-dependent necrotic cell death through calpain-triggered mitochondrial depolarization and reactive oxygen species-mediated inhibition of nuclear factor-κB activity. Chem. Res. Toxicol. 20, 406–415.

Yih, L.H., Lee, T.C., 2000. Arsenite induces p53 accumulation through an ATM-dependent pathway in human fibroblasts. Cancer Res. 60, 6346–6352.

Yin, Z., Milatovic, D., Aschner, J.L., Syversen, T., Rocha, J.B.T., Souza, D.O., Sidoryk, M., Albrecht, J., Aschner, M., 2007. Methylmercury induces oxidative injury, alterations in permeability and glutamine transport in cultured astrocytes. Brain Res. 1131, 1–10.

Youle, R.J., Strasser, A., 2008. The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell. Biol. 9, 47–59.

Zahn, T., Arnold, H., Braunbeck, T., 1996. Cytological and biochemical response of R1 cells and isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) to subacute *in vitro* exposure to disulfoton. Exp. Toxicol. Pathol. 48, 47–64.

Zaja, R., Munić, V., Klobučar, R.S., Ambriović-Ristov, A., Smital, T., 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat. Toxicol. 90, 322–332.

Zalups, R.K., 2000. Molecular interactions with mercury in the kidney. Pharmacol. Rev. 52, 113–143.

Zalups, R.K., Ahmad, S., 2003. Molecular handling of cadmium in transporting epithelia. Toxicol. Appl. Pharmacol. 186, 163–188.

Zangi, R., Filella, M., 2012. Transport routes of metalloids into and out of the cell: a review of the current knowledge. Chem. Biol. Interact. 197, 47–57.

Zelikoff, J.T., Bowser, D., Squibb, K.S., Frenkel, K., 1995. Immunotoxicity of low level cadmium exposure in fish: an alternative animal model for immunotoxicological studies. J. Toxicol. Environ. Health 45, 235–248.

Zelikoff, J.T., Raymond, A., Carlson, E., Li, Y., Beaman, J.R., Anderson, M., 2000. Biomarkers of immunotoxicity in fish: from the lab to the ocean. Toxicol. Letters. 112–113, 325–331.

Zhang, Q., Zeng, G., Chen, G., 2015. The effect of heavy metal-induced oxidative stress on the enzymes in white rot fungus *Phanerochaete chrysosporium*. Appl. Biochem. Biotechnol. 175, 1281–1293.

Zhao, S., Tsuchida, T., Kawakami, K., Shi, C., Kawamoto, K., 2002. Effect of As_2O_3 on cell cycle progression and cyclins D1 and B1 expression in two glioblastoma cell lines differing in p53 status. Int. J. Oncol. 21, 49–55.

Zhaobao, Y, Eunsook, L., Mingwei, N., Haiyan, J., Milatovica, D., Rongzhua, L., Farinac, M., Rochad, J.B.T., Aschner, M., 2011. Methylmercury-induced alterations in astrocyte function are attenuated by Ebselen. Neurotoxicology 32, 291–299.

Zheng, G.H., Liu, C.M., Sun, J.M., Feng, Z.J., Cheng, C., 2014. Nickel-induced oxidative stress and apoptosis in *Carassius auratus* liver by JNK pathway. Aquat. Toxicol. 147, 105–111.

Zheng, Y., Peng, L.M., You, F., Zou, Y.X., Zhang, P.J., Chen, S.L., 2015. Establishment and characterization of a fish-cell line from the brain of Japanese flounder *Paralichthys olivaceus*. J. Fish Biol. 87, 115–122.

Zikić, R.V., Stajn, A.S., Pavlović, S.Z., Ognjanović, B.I., Saićić, Z.S., 2001. Activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of goldfish (*Carassius auratus gibelio* Bloch.) exposed to cadmium. Physiol. Res. 50, 105–111.

Zohouri, M.A., Pyle, G.G., Wood, C.M., 2001. Dietary Ca inhibits waterborne Cd uptake in Cd-exposed rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. C 130, 347–356.

Zucchi, S., Corsi, I., Luckenbach, T., Bard, S.M., Regoli, F., Focardi, S., 2010. Identification of five partial ABC genes in the liver of the Antarctic fish *Trematomus bernacchii* and sensitivity of ABCB1 and ABCC2 to Cd exposure. Environ. Pollut. 158, 2746–2756.

Zupanc, G.K.H., Clint, S.C., 2003. Potential role of radial glia in adult neurogenesis of teleost fish. Glia 43, 77–86.